

**MECHANISTIC SIGNIFICANCE OF
HETEROTROPIC COOPERATIVITY EFFECTS IN THE
LIVER ALCOHOL DEHYDROGENASE SYSTEM**

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Lund 1985



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Abstract The catalytic reaction mechanism of horse liver alcohol dehydrogenase (LADH) has been investigated with particular reference to the existence and catalytic significance of heterotropic cooperativity effects. Results presented in this thesis provide no evidence indicating that the enzyme shows half-site reactivity or that it is dependent on any cooperative subunit interactions. The binding properties and kinetic behaviour of the enzyme can be explained in terms of equivalent and independent catalytic sites. Enzymic aldehyde reduction is shown to proceed by a random-order mechanism for ternary-complex formation despite the fact that the reaction is governed by a compulsory-order type of rate equation. Aldehydes show a significant affinity for the substrate-binding site in free enzyme but they are not bound to the catalytic zinc ion in these binary complexes. Complex formation with NADH increases the affinity of the enzyme for aldehydes due to hydrophobic substrate-coenzyme-site interactions and leads to inner-sphere coordination of the substrates to the metal ion. Alcohols are shown to combine to catalytic zinc also in the absence of coenzyme and they exhibit ionization properties analogous to those of zinc-bound water. NAD⁺ binding causes a pK_a perturbation which ensures that alcohols are bound predominantly as alcoholate ions at physiological pH. This activation of the substrate facilitates the subsequent step of hydride transfer to NAD⁺.			
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av

PIA ANDERSSON

Fil. kand. , M

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CONTENTS

PUBLICATIONS	4
1. INTRODUCTION	5
1.1 Enzyme	5
1.2 Enzyme structure	6
1.3 Enzyme mechanism	9
1.4 Aims of the present work	13
2. KINETIC SIGNIFICANCE OF SUBUNIT INTERACTIONS	16
3. COOPERATIVITY IN THE BINDING OF COENZYME AND ALDEHYDES	17
4. KINETIC MECHANISM	19
5. EFFECT OF pH ON LIGAND BINDING TO CATALYTIC ZINC	22
6. COOPERATIVITY IN THE BINDING OF COENZYME AND ALCOHOLS	25
7. GENERAL CONCLUSIONS	30
8. ACKNOWLEDGEMENTS	32
9. REFERENCES	33

PUBLICATIONS

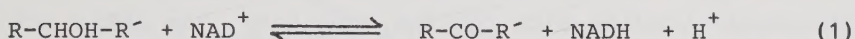
This thesis summarizes the contents of the following publications, which will be referred to by their Roman numerals:

- I. Kinetic Equivalence of the Subunits of Liver Alcohol Dehydrogenase
Pia Andersson and Gösta Pettersson (1982)
Eur. J. Biochem. 122 , 559-568
- II. Effect of pH on Pyrazole Binding to Liver Alcohol Dehydrogenase
Pia Andersson, Jan Kvassman, Anders Lindström, Bertil Oldén and Gösta Pettersson (1981)
Eur. J. Biochem. 114 , 549-554
- III. Synergism between Coenzyme and Aldehyde Binding to Liver Alcohol Dehydrogenase
Pia Andersson, Jan Kvassman, Bertil Oldén and Gösta Pettersson (1983)
Eur. J. Biochem. 133 , 651-655
- IV. Catalytic Significance of Binary Enzyme-Aldehyde Complexes in the Liver Alcohol Dehydrogenase Reaction
Pia Andersson, Jan Kvassman, Bertil Oldén and Gösta Pettersson (1984)
Eur. J. Biochem. 139 , 519-527
- V. Synergism between Coenzyme and Alcohol Binding to Liver Alcohol Dehydrogenase
Pia Andersson, Jan Kvassman, Bertil Oldén and Gösta Pettersson (1984)
Eur. J. Biochem. 144 , 317-324

1. INTRODUCTION

1.1 Enzyme

Redox reactions involving nicotinamide adenine dinucleotides as electron donors/acceptors are of outstanding metabolic importance and the structure and function of the enzymes (dehydrogenases) which catalyze such reactions has been at the focus of biochemical interest since the birth of modern enzymology. One of the most studied enzymes of this category is the alcohol dehydrogenase which occurs in the liver of mammals. This enzyme acts on the -CHOH group of donors according to the equation



i.e. shows catalytic activity towards primary alcohols, secondary alcohols and hemiacetals as well as their oxidized aldehyde or ketone counterparts.

Liver alcohol dehydrogenase (which will be abbreviated LADH) was purified to the crystalline state by Bonnichsen et al. in 1948 (1) and was subjected to pioneering enzymological studies by Theorell and coworkers during the following decade. Since then, the structural and catalytic properties of LADH have been investigated by a large number of research groups and by a great variety of experimental approaches and techniques. The fact that the enzyme contains catalytically essential zinc ions has rendered LADH an interesting object of study also as a representative of the biochemically important group of metallo-enzymes. The vast amount of information which is now available on the structural, mechanistic and physico-chemical properties of LADH has recently been summarized in several excellent review articles (2,3,4).



It may suffice, therefore, to summarize here the present state of knowledge on the structure and function of LADH with regard to problems considered in this thesis.

1.2 Enzyme structure

LADH is a dimer consisting of two structurally identical subunits (5). Each subunit contains two zinc ions, one of which is known to be catalytically essential (2). Crystallographic results presented by Brändén and coworkers (6) have shown that the catalytic zinc ion is located at the bottom and junction of two deep perpendicular pockets which constitute the active-site region of the enzyme subunit (Fig.1-2). One of these pockets functions as a hydrophobic binding site for the substrate, the other accommodates the nicotinamide portion of the coenzyme with its reactive part in close proximity to catalytic zinc.

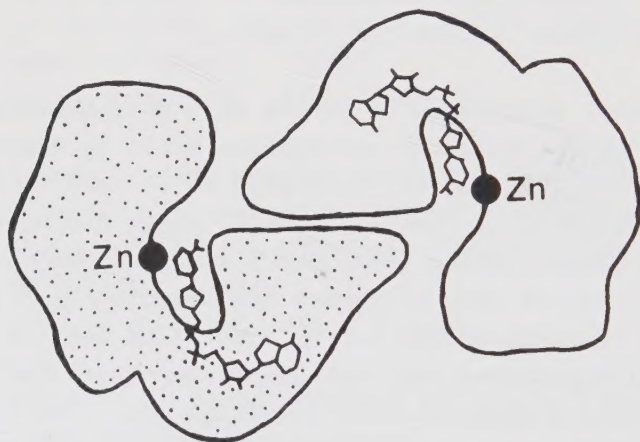


Fig. 1 A schematic diagram of the horse liver alcohol dehydrogenase dimer, showing the position of bound NAD^+ .

In free enzyme, the active-site zinc ion is tetra-coordinate. The protein provides three ligands for the metal ion and the fourth coordination position is occupied by a water molecule which protrudes into the substrate-binding pocket. This zinc-bound water molecule appears to be displaced by substrate in the productive enzyme-coenzyme-substrate complex formed during catalysis (7). Coordination of the substrate to catalytic zinc brings bound substrate and coenzyme in adequate proximity and orientation for direct hydride transfer to take place (Fig.2) and may be catalytically favourable also in other respects.

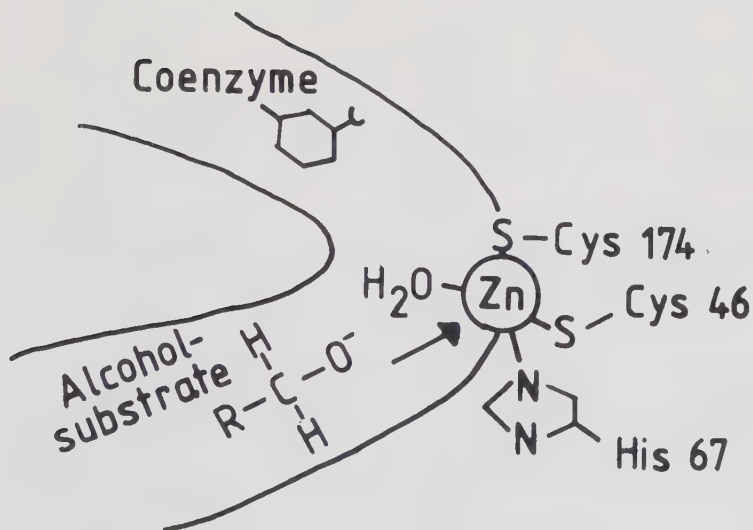


Fig. 2 A schematic representation of the hydrophobic barrel constituting the substrate-binding site of the LADH subunit.

Crystallographic studies have provided clear evidence that coenzyme binding triggers conformational changes of the enzyme subunit such that the nicotinamide-binding pocket becomes closed. Subsequent substrate-binding causes a further tightening of the enzyme structure and leads to extensive dehydration of the active-site region (4,8,9). The catalytic ternary-complex interconversion step, therefore, appears to take place in an essentially anhydrous environment, and attention has been drawn to a structural arrangement of hydrogen bonds which may serve as a relay for proton transfer from the catalytic center to solution (Fig.3).

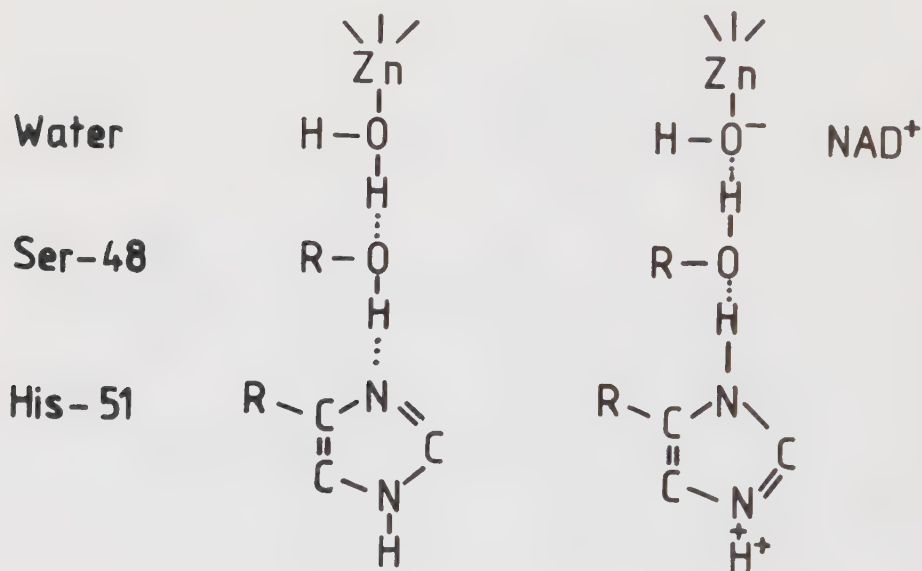


Fig. 3 Schematic diagram illustrating the hydrogen bond system, which is likely to mediate proton transfer from the catalytic center to solution.

1.3 Enzyme mechanism

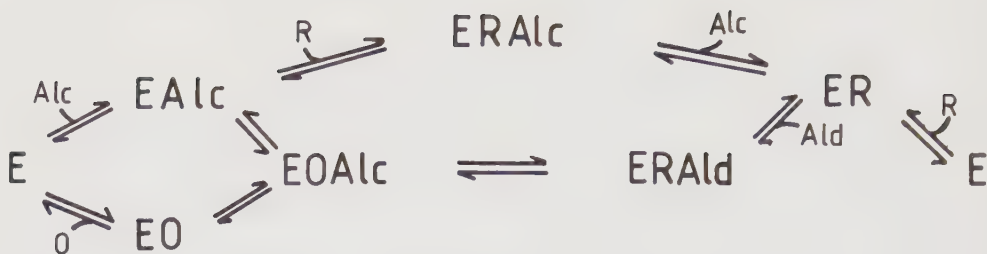
1.3.1 Kinetic mechanism

LADH, like most other dehydrogenases operates by a ternary-complex mechanism (20) i.e. the catalytic reaction between substrate and coenzyme takes place in a ternary enzyme-coenzyme-substrate complex. In principle, the formation of such a complex may proceed by a random order of binding of the two ligands. It was early shown, however, that the steady-state rate behaviour of LADH at non-inhibitory substrate concentrations is consistent with a compulsory-order mechanism in which coenzyme binding precedes substrate binding, and product dissociation precedes coenzyme dissociation.



Scheme 1 (E=LADH R=NADH S=aldehyde P=alcohol O=NAD⁺)

Later studies have established that Scheme 1 is insufficient to explain all aspects of the kinetic behaviour of LADH. At high substrate concentrations, the oxidation of alcohols may exhibit deviations from Michaelis-Menten kinetics indicative of the formation of non-productive enzyme-NADH-alcohol complexes (10,11). With certain substrates, these non-productive ternary complexes may dissociate to enzyme-alcohol complexes that may bind coenzyme and so contribute to the catalytic reaction flow (12,13). Observations of that kind led Dalziel to propose in 1972 the following kinetic scheme:



Scheme 2

Other studies have revealed that the enzyme shows aldehyde dehydrogenase (dismutase) activity (14-17), which means that ternary enzyme-NAD⁺-aldehyde complexes may also be of catalytic significance.

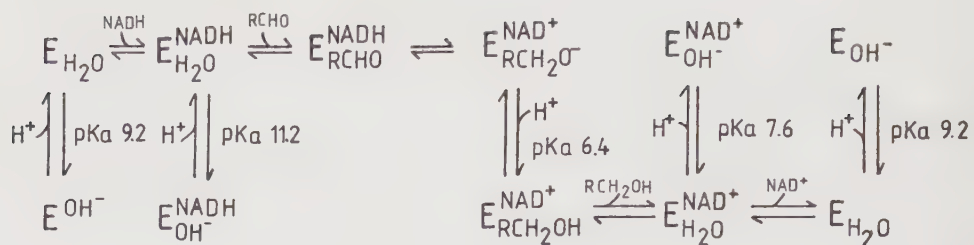
Further evidence that LADH catalyzed reactions are not strictly compulsory-order has come from isotope exchange measurements (18,19). Contributions from pathways involving binary enzyme-substrate complexes could be detected at high substrate concentrations. This has led to general acceptance of the idea that LADH operates by an "effectively ordered" or "preferred-order" mechanism. According to this idea, the alternative pathway involving primary substrate binding does exist, but the pathway is a minor one which contributes negligibly to the catalytic reaction-flow under conditions where the enzyme obeys Michaelis-Menten kinetics.

1.3.2 Proton transfer mechanism

Kinetic, spectroscopic and crystallographic studies have provided much detailed information on the binding interactions of LADH with substrate and coenzyme (4,9,21-23). Such results consistently indicate that catalytic hydride ion transfer occurs directly between bound substrate and coenzyme in the productive ternary complex formed during catalysis. According to Eqn(1), the catalytic reaction also involves a step of proton exchange between the alcohol substrate/product and solution. The mechanism for this catalytic proton transfer step has been a matter of intense debate, and several more or less controversial interpretations of the pH dependence of the catalytic reaction have been presented (21-23). Much of the later debate has focused on the effect of pH on coenzyme binding and on the ionization properties of the water molecule which is bound to catalytic zinc in the absence of substrate.

There are several lines of evidence showing that NAD^+ binding drastically increases the acidity of zinc-bound water (2,24,25), suggesting that catalytic proton exchange with solution may occur already in the binary enzyme-coenzyme complex. Most mechanistic proposals, therefore, have attributed a catalytically crucial function to zinc-bound water in its ionized state (2,26,27).

A fundamentally different view was expressed by Kvassman & Pettersson, who concluded in 1980 from a systematic series of pH dependence studies that coenzyme-induced proton release from zinc-bound water represents a side-reaction which is not on the main catalytic pathway (28). They proposed the following reaction mechanism:



Scheme 3

according to which catalytic proton transfer occurs at the ternary-complex level in an obligatory step of alcohol/alcoholate ion equilibration of the zinc-bound substrate. Scheme 3 provides a reasonable explanation for all main effects of pH on LADH catalysis, and is attractive also because of its simplicity. The pH dependences of the catalytic reaction is attributed to analogous steps of ionization of zinc-bound water and alcohol substrate.

1.3.3 Subunit cooperativity

Equilibrium binding studies and steady-state kinetic measurements have failed to provide evidence for any pre-existing or induced non-equivalence of the two subunits of the LADH molecule.

In 1970, however, Bernhard et al. (29) reported that the enzymic reduction of aromatic aldehydes shows a biphasic transient time course under single turnover conditions, each phase corresponding approximately to consumption of one-half of the limiting reagent (enzyme, coenzyme or substrate). The interpretation of these observations was that the biphasic rate behaviour reflects ligand-dependent subunit interactions resulting in apparent "half-site" reactivity of the enzyme.

The half-sites reactivity hypothesis has received support by several authors (30-33), but alternative interpretations of the biphasic transient rate behaviour of LADH have also been put forward (34-36). In particular, Pettersson in 1976 (35) presented a theoretical analysis showing that the transient-state kinetics of LADH catalysis are qualitatively consistent with a compulsory order mechanism involving equivalent and independent sites.

1.4 Aims of the present work

If LADH operates by an effectively ordered mechanism with coenzyme binding preceding substrate binding, then there must be some specific reason why this binding order is preferred. Coenzyme binding might either drastically favour or be drastically disfavoured by the interaction of enzyme with the substrate. This would imply that there is a most significant (heterotropic) cooperativity in the

processes of substrate and coenzyme binding to the enzyme. Since the NADH, NAD^+ and alcohol binding steps are strongly pH dependent, and since alcohol substrates are ionizable, the substrate/coenzyme interactions could involve also the proton cooperatively bound as a third ligand.

The structural requisites for a kinetic and/or thermodynamic synergism between substrate and coenzyme binding are undoubtedly at hand. The ligands are bound at adjacent sites (Fig.2) and complex formation at one site would actually be expected to affect the binding properties of the other, particularly since at least coenzyme binding is known to trigger enzyme conformational changes. As may be noted from Fig.1, further, the substrate binding pocket at the catalytic center of one subunit is constituted by protein residues from both subunits. This structural arrangement might well allow for the appearance of ligand-induced cooperative subunit interactions during catalysis.

When the work summarized in this thesis was initiated, positively as well as negatively cooperative effects of NADH or NAD^+ on the binding of various substrate analogues had been well documented (2,37). Little was known, however, about the existence, sign and strength of the cooperative interactions of coenzymes with actual substrates, despite the fact that such interactions relate directly to the kinetics and thermodynamics of formation of the catalytically productive ternary-complexes and hence are of obvious mechanistic interest. Furthermore, the identity and catalytic function of the ionizing groups which account for the pH dependence of LADH catalysis remained uncertain and the half-site reactivity hypothesis was still considered viable.

Investigations summarized in this thesis were initiated to contribute to the elucidation of some of the fundamental mechanistic problems considered above, with particular reference to the existence and catalytic significance of heterotropic cooperativity effects. Paper I concerns the subunit-cooperativity problem and the half-sites reactivity hypothesis. Results presented in Paper II relate to the binding characteristics of ionizing ligands, and hence indirectly to the effect of coenzymes on alcohol binding. Papers III-V finally, deal with the synergism between coenzymes and substrates in their interactions with the enzyme.

2. KINETIC SIGNIFICANCE OF SUBUNIT INTERACTIONS

Experimental tests of the transient state kinetic relationships derived by Pettersson (35) for the mechanism in Scheme 1 led Kvassman & Pettersson (38) to conclude that there is no evidence for any kinetically significant subunit cooperativity in the LADH system. This conclusion was strongly criticized by Dunn and coworkers (32) who pointed out that the theory applied by Kvassman & Pettersson was based on some simplifying assumptions such as neglectance of the absorption differences between different NAD^+ -containing enzyme species. Dunn et al. attempted to introduce corrections for such absorption differences applicable to experimental data obtained by the "catalytic-suicide" method of Bernhard et al. (29). They found that corrected transient-state kinetic amplitude values for the enzymic reduction of a series of benzaldehyde derivatives were in substantial disagreement with the ordered mechanism proposed by Kvassman & Pettersson (38) and more consistent with the "half-site" reactivity proposal of Bernhard et al. (29). Additional experimental data leading to similar conclusions were later reported by other authors (33,39).

It was of obvious mechanistic and methodological importance to resolve once and for all the 10-year-old controversy regarding the kinetic equivalence of the LADH subunits. In Paper I, therefore, relationships were derived which clarify the implications of the Pettersson theory with respect to the precise magnitude of amplitudes observed in catalytic suicide experiments.

After determination of some critical spectral data, the extended theory was applied to the LADH system. The results showed that reported transient-state amplitude data for aldehyde reduction are in remarkably excellent quantitative agreement with the predictions of an ordered mechanism involving equivalent and independent sites. There is no reason to believe that the ligation state at one subunit in the LADH molecule has any significant influence on the binding or catalytic properties of the second subunit.

3. COOPERATIVITY IN THE BINDING OF COENZYME AND ALDEHYDES

Dunn et al. (40) were the first to present direct evidence that substrates combine to catalytic zinc in the productive ternary-complexes formed during LADH catalysis. They found that trans-N,N-dimethylaminocinnamaldehyde (DACA) forms a chromophoric complex with LADH in the presence of NADH, while no such complex was formed in the presence of NAD^+ or in the absence of coenzymes. Hence they concluded that NADH plays a specific effector role in the chemical activation of aldehyde substrates.

Dunn (37) in a later interpretation of the experimental results suggested that NADH binding drastically increases the affinity of the enzyme for aldehydes thus conveying a kinetically discernable order to the binding of coenzyme and substrate. The idea that NADH promotes the binding of aldehydes seemed most reasonable considering that neither binding studies, nor kinetic measurements, had provided any reliable evidence for the

formation of binary enzyme-aldehyde complexes. No attempts had been made, however, to estimate the actual magnitude of the synergistic effects of coenzymes on the equilibrium binding of aldehydes.

The investigation in Paper III was undertaken to obtain such information. To this end, we determined the affinity of the enzyme for some aldehydes by spectroscopic methods using reporter ligands such as auramine O and 2,2'-bipyridine.

The results showed that aldehydes such as DACA and benzaldehyde exhibit a significant, but pH-independent, affinity for the substrate-binding site in free enzyme and that the enzyme-DACA complex is non-chromophoric. These observations and the fact that aldehyde binding shows no competition with 2,2'-bipyridine binding indicate that aldehydes are not bound to the catalytic zinc ion in the binary complex.

Complex formation between enzyme and NADH stabilizes the binding of aldehydes, but only by a factor of approximately 10. This effect can be attributed to hydrophobic substrate-coenzyme-site interactions. Simultaneous occupation of the coenzyme-binding and substrate-binding sites results in a closer packing of the enzyme and in dehydration of the active site, which facilitates metal-coordination of the aldehyde substrate.

LADH shows a dismutase activity towards most aldehydes, i.e. may oxidize aldehydes to their carbonic acid counterparts in the presence of NAD^+ (11). Aldehyde binding to the enzyme- NAD^+ complex, therefore, can be anticipated to result more or less extensively in direct coordination of the substrate to catalytic zinc. Our attempts in Paper III to provide evidence for such metal-coordination of aldehyde ligands were unsuccessful; the affi-

nity of the enzyme- NAD^+ complex for DACA and benzaldehyde showed no dependence on the protonation state of zinc-bound water. It has recently been shown, however, that the chromophoric properties of the enzyme- NAD^+ -DACA complex are strongly pH-dependent and indicative of metal-coordination of DACA at low pH. The latter results, combined with those in Paper III, lead to the conclusion that coordination of aldehydes to zinc per se does not contribute much to the net energetics of substrate binding or to the stabilization of the complex. Variation of pH has essentially no effect on the affinity of the enzyme for aldehydes, but drastically affects the extent to which the bound aldehyde becomes directly coordinated to zinc. There appears to be an equilibrium distribution of bound aldehydes between the inner and outer coordination spheres of the zinc ion depending on how well the aldehyde can compete with the water molecule (hydroxyl ion) for inner sphere coordination to the metal.

4. KINETIC MECHANISM

Results in Paper III confirmed that NADH increases the affinity of LADH for aldehyde substrates, but also established that binary enzyme-aldehyde complexes do form over experimentally accessible concentration ranges. This means that the apparent kinetic insignificance of the enzyme-aldehyde pathway for ternary-complex formation according to Scheme 4 cannot be related to the stabilizing effect of coenzyme on substrate binding (substrate stabilizes coenzyme binding to exactly the same extent). Instead, it might reflect cooperative effects of coenzyme and substrate on the kinetics of their associa-

tion to the enzyme.



Scheme 4

The investigation in Paper IV was carried out to test this idea. We used DACA as substrate since this ligand is chromophoric and forms a productive complex with rather low catalytic reactivity. Furthermore, detailed kinetic data for formation of the enzyme-NADH-DACA complex by the enzyme-NADH pathway in Scheme 4 were already available (40,41), such that our studies could focus on characterization of the kinetics of ligand binding in the enzyme-aldehyde pathway. The results showed that there is no effect of DACA on the association of NADH or vice versa. The ten-fold stabilization of the ternary complex reflects exclusively effects on dissociation rates of the ligands.

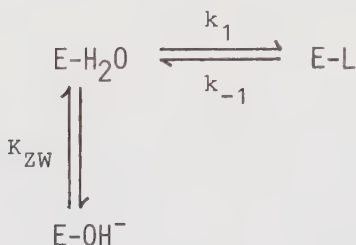
The observation that DACA and coenzyme association rates are not cooperatively affected provided evidence against the idea that the enzyme-coenzyme pathway for ternary-complex formation in Scheme 4 is kinetically favoured over the alternative pathway involving intermediate formation of a binary enzyme-aldehyde complex. Reaction flow analysis of Scheme 4 based on data obtained with

DACA as the substrate showed that both pathways are catalytically significant and that reaction flow via the enzyme-aldehyde complex may even predominate at high DACA concentrations. Since the kinetic characteristics accounting for this flow behaviour of the system are likely to obtain with aldehydes in general, it was concluded that LADH must be assumed to catalyze aldehyde reduction by a random-order mechanism. The latter conclusion was in obvious conflict with the widely held view that LADH operates by an effectively ordered mechanism (42-44) and might seem to be inconsistent also with the fact that aldehyde reduction invariably has been found to be governed by a compulsory-order type of rate equation (10,43,45,46).

The theoretical analysis presented in Paper IV resolved this apparent controversy, however, and provided an explanation for the rate behaviour of LADH. If aldehyde reduction by the random pathway in Scheme 4 shows some fundamental kinetic characteristics agreeing with those observed for DACA reduction, then the catalytic reaction will be governed by a compulsory-order type of rate equation irrespective of what pathway is actually preferred. Our demonstration that the rate equation not necessarily gives any reliable information on the ligand binding order in ternary-complex systems has some important implications as to the rate behaviour of enzyme reactions in general.

5. EFFECT OF pH ON LIGAND BINDING TO CATALYTIC ZINC

The rate of displacement of a metal-coordinated hydroxide ion can be anticipated to be negligibly low compared to the rate of displacement of metal-bound neutral water. Ligands (L) which combine to catalytic zinc in LADH with displacement of zinc-bound water, therefore, will interact predominantly with the enzyme species that contains zinc-bound water in its unionized state

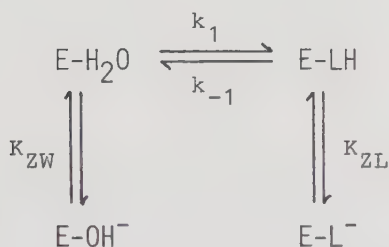


Scheme 5

i.e. kinetic and equilibrium constants for such complex formation will usually show a pH dependence reflecting the ionization state of zinc-bound water.

Several inhibitory reagents (and aldehydes in their interaction with the enzyme-NADH complex) are known to be directly bound to catalytic zinc with displacement of zinc-bound water. Examination of the effect of pH on the binding of such reagents has made it possible to estimate the acid dissociation constant (K_{ZW}) for zinc-bound water in the absence or presence of bound coenzyme. The pK_a values thus obtained are given in Table 1 and agree with those controlling anion (47) and coenzyme binding (41) to the enzyme (Scheme 3).

Scheme 5 applies to the binding of non-ionizing ligands to catalytic zinc in LADH. As pointed out by Kvassman & Pettersson, however, zinc-coordination of ionizing ligands (LH) must be expected to depend not only on the ionization state of zinc-bound water but also on the ionization properties of the ligand itself.



Scheme 6

Kinetic and equilibrium constants for complex formation according to Scheme 6 will be given by

$$k_{\text{on}} = \frac{k_1}{1 + K_{ZW} / (\text{H}^+)} \quad (2)$$

$$k_{\text{off}} = \frac{k_{-1}}{1 + K_{ZL} / (\text{H}^+)} \quad (3)$$

$$\frac{k_{\text{off}}}{k_{\text{on}}} = \frac{k_{-1}}{k_1} \cdot \frac{1 + K_{ZW} / (\text{H}^+)}{1 + K_{ZL} / (\text{H}^+)} \quad (4)$$

The validity of Scheme 6 and Eqns. (2-4) was tested in Paper II by examination of the effect of pH on pyrazole binding to LADH. The inhibitor pyrazole forms a chromophoric complex indicative of zinc-coordination of the ligand, and its binding can be readily monitored by spectrophotometric methods. Such binding studies established that complex formation between enzyme and pyrazole shows the expected dependence on the protonation state of the ionizing group identified as zinc-bound water, as well as a dependence on an ionization step reflecting deprotonation of pyrazole itself.

Besides corroborating the spectroscopic evidence for zinc-coordination of pyrazole (recently confirmed by crystallographic studies (48)) and the validity of the pK_a values assigned to zinc-bound water (Table 1), results in Paper II provided evidence for a close analogy between pyrazole and alcohols in their binding interactions with the enzyme. Not only does complex formation between enzyme and these ligands show a qualitatively agreeing pH dependence, but the ionization properties of the bound ligands are also analogously affected by the coenzymes. These observations lend support to the proposal of Kvassman & Pettersson that proton dissociation from zinc-bound alcohols represents a crucial reaction step controlling substrate binding to the enzyme (Scheme 6).

The main objective of the study in Paper II, however, was to characterize the binding of a chromophoric reagent that could be used as a reporter ligand for examination of binding interactions which are not associated with detectable spectral changes. Using pyrazole data from Paper II, Kvassman et al. managed to determine pK_{ZL} values (Scheme 6) for a series of enzyme- NAD^+ -alcohol complexes (49). They found that pK_{ZL} values for the enzymic complexes are linearly correlated with the pK_a

values of the free alcohol ligands (Table 1). The latter finding provides crucial evidence in favour of the mechanism in Scheme 3 by establishing that the zinc-bound alcohol forms part of the ionizing system that accounts for the pK_{ZL} dependence of alcohol binding to LADH. In fact, as argued in the discussion of Paper II, it seems reasonable only to attribute the kinetically determined pK_{ZL} values to ionization of the zinc-bound alcohols themselves.

6. COOPERATIVITY IN THE BINDING OF COENZYME AND ALCOHOLS

Aldehydes do not compete successfully with water for the inner coordination sphere of catalytic zinc in the absence of coenzymes (2,4). Alcohols, however, show an intrinsic affinity for metal ions comparable to that of water and might well combine to catalytic zinc also in their interaction with free enzyme. If so, metal coordination of the alcohol should increase its acidity such that the corresponding pK_a values might be estimated from binding studies carried out over an experimentally readily accessible pH range (6-10).

Our examination of coenzyme/substrate cooperativity effects relating to the formation of productive enzyme- NAD^+ -alcohol complexes in Paper V focused primarily on the binding and bonding interactions of alcohols with free enzyme. Using auramine O and 2,2'-bipyridine as reporter ligands, we found that alcohols do combine to the substrate-binding site in free enzyme and that such

complex formation leads to inner-sphere coordination of the alcohol to catalytic zinc. The latter conclusion was corroborated by the observation that alcohol binding to free enzyme exhibits a pH dependence consistent with Scheme 6 and Eqns. (2-4) i.e. entirely analogous to that of alcohol binding to the enzyme-NAD⁺ complex (49). Furthermore, estimates of pK_{ZL} values for the ionization of a series of zinc-bound alcohols were found to show a linear Brönstedt correlation with the pK_a values of the free alcohol ligands. This would seem to definitely establish the validity of our interpretation of the experimental data in terms of Scheme 6.

According to Scheme 6, the affinity of LADH for alcohols is regulated by three different processes: the ionization of zinc-bound water, the ionization of the zinc-bound alcohol and the actual step of complex formation between the unionized alcohol and the binding protonation state of the enzyme. Results presented in Paper V and elsewhere (28,41,49,50) establish that coenzyme binding affects each one of these processes. The binding of NAD⁺ and alcohols to LADH, therefore, shows a comparatively complex synergistic pattern over the pH range 6-10, illustrated by the equilibrium data in Fig. 4 of Paper V.

This synergistic pattern can be rationalized in simple terms, however, when related to the effects of NAD⁺ on the individual processes in Scheme 6. Thus, results in Paper V are consistent with previously reported effects of coenzymes on the ionization properties of zinc-bound water and provide clear evidence that the ionization properties of zinc-bound alcohols are similarly affected (Table 1). Furthermore, equilibrium effects of NAD⁺ on the actual step of (neutral) alcohol binding agree with the observed effects of NADH on aldehyde binding (III), i.e. the affinity of the enzyme for substrates is basi-

cally increased about ten-fold on binding of the coenzyme. This lends support to the proposal in Paper III that hydrophobic interactions (reflecting the thermodynamic advantage of simultaneous occupation of the adjacent substrate- and coenzyme-binding sites) provide a basic contribution to synergistic stabilization of the productive ternary complexes.

In Paper III we found that enzyme-NADH-aldehyde complexes are stabilized by decreased rates of dissociation of coenzyme and substrate. Results in Paper V show that the synergism between NAD^+ and alcohol binding reflects effects on both dissociation and association rates of the ligands. Our interpretation of the latter observation is that the alcohol hydroxyl group, but not the aldehyde carbonyl group, participates in hydrogen bonding interactions with the enzyme.

This adds weight to the crystallographic evidence (9) indicating that the system of hydrogen bonds depicted in Fig.3 may play an essential mechanistic role as a mediator of proton transfer from alcohol substrates to solution. As pointed out in Paper V, the system of hydrogen bonds does not seem to contribute significantly to the net energetics of alcohol binding. The functional role of the proton relay system, therefore, should be related to a kinetic increase of the proton transfer rate rather than to a thermodynamic increase in acidity of the enzyme-bound substrate.

Kvassman & Pettersson in their mechanistic proposal (23) assumed that alcohol substrates ionize with pK_a values below 7 in the productive ternary-complexes. This drastic increase in acidity of the substrate was tentatively attributed to the combined effects of metal-coordination of the alcohol and of electrostatic interactions

with bound NAD^+ . Results in Paper V confirm that both effects are at hand and provide direct information about their relative magnitude (Table 1). Coordination to catalytic zinc in the binary enzyme complexes decreases the pK_a for ionization of alcohols by 5-6 units. NAD^+ binding causes a further decrease in pK_a by 2-3 units. The latter effect appears to be of particular mechanistic interest since it renders alcohol ligands ($\text{pK}_a < 6.4$) more acid than zinc-bound water (pK_a 7.6) in the presence of NAD^+ . This means that zinc-bound water is predominantly unionized in the substrate-binding species at physiological pH, thus facilitating ligand exchange at the metal ion. Zinc-bound alcohols, however, will be predominantly ionized in the productive ternary-complexes, which facilitates the subsequent step of catalytic hydride transfer to NAD^+ .

Alcohol	Estimated pK_a value for			pK_a shift induced by	
	Alc	E-Alc	E-NAD ⁺ -Alc	Enzyme	NAD ⁺
Trifluoroethanol	12.4	7.8	4.3	4.6	3.5
2,2'-Dichloroethanol	12.9	8.0	4.5	4.9	3.5
2-Chloroethanol	14.3	8.6	5.4	5.7	3.2
Benzylalcohol	15.1	9.2	6.4	5.9	2.8
Water	15.6	9.2	7.6	6.4	1.6

Table 1 pK_a values determined for alcohol ionization in solution and in binary or ternary enzyme-alcohol complexes.

7. GENERAL CONCLUSIONS

Since LADH is a dimer consisting of two catalytically active subunits, it is of primary mechanistic interest to decide whether or not the two subunits operate independently of each others. Results summarized in this thesis provide a clear answer in that respect. There is no evidence indicating that the catalytic activity of LADH is dependent on, or significantly affected by, cooperative interactions of one subunit with the other. The binding properties and kinetic behaviour of the enzyme can be excellently accounted for in terms of equivalent and independent catalytic sites.

The fact that LADH catalysis conforms to a compulsory-order type of rate equation under Michaelis-Menten conditions has been generally taken to indicate that there is an inherent kinetic preference in the coenzyme and substrate binding order. The present results have failed to provide evidence in that direction. On the contrary, data reported in Paper IV lead to the conclusion that enzymic aldehyde reduction must be assumed to proceed by a random-order mechanism for ternary-complex formation, despite the fact that a compulsory-order type of rate-equation obtains. The rate behaviour of LADH cannot be directly related to any cooperativity effects in the substrate and coenzyme binding processes.

The coenzyme/substrate cooperativity documented by the results in Paper III and V would seem to be mechanistically interesting in other respects, however. The observed synergistic stabilization of productive ternary-complexes at neutral pH will tend to decrease the K_m -values for alcohol and aldehyde substrates, an effect which might be of physiological importance. In the case of aldehydes, the presence of bound coenzyme appears to be

required to force the substrate on to catalytic zinc. In the case of alcohols, NAD^+ binding is essential to cause the pK_a perturbation which ensures that alcohols are bound predominantly as alcoholate ions at physiological pH.

Results in Paper V are particularly informative from a mechanistic point of view. They provide crucial evidence in favour of the mechanistic proposal of Kvassman & Pettersson (Scheme 3) by establishing that enzyme-bound alcohols do combine to catalytic zinc and do exhibit ionization properties analogous to those of zinc-bound water. The pK_a values of zinc-bound water in different enzymic species have been satisfactorily explained in terms of the enzymic metal-site structure and of electrostatic field effects of the bound coenzymes (51). Data in Paper V establish that the increased acidity of enzyme-bound alcohols can be analogously explained.

Consequently, we now seem to have a comparatively good understanding of some fundamental factors contributing to the chemical activation of substrates during LADH catalysis, as well as of the role of coenzymes in this process.

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Kinetic Equivalence of the Subunits of Liver Alcohol Dehydrogenase

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1. The kinetics of two-substrate enzyme reactions proceeding by an ordered ternary-complex mechanism have been analyzed theoretically with respect to the precise magnitude of amplitudes of the two slowest transients governing such reactions under single-turnover conditions. Relationships derived have been applied to the liver alcohol dehydrogenase system for detailed interpretation of amplitudes of the biphasic absorbance changes associated with the enzymic reduction of aromatic aldehydes in catalytic-suicide experiments.

2. Amplitudes of the slow phase 328–330-nm absorbance changes occurring during the enzymic reduction of various *para*-substituted benzaldehydes by NADH or NAD²H correspond to 6–26% of the total absorbance change under conditions of substrate and coenzyme saturation. Saturation values of amplitudes determined in the present and previous investigations agree within 2% of the total absorbance change with those calculated theoretically for an ordered mechanism of enzyme action at equivalent sites. In particular, amplitudes observed exhibit the expected sensitivity to electronic substituent and deuterium isotope effects.

3. Enzyme recycling, aldehyde binding, and alcohol desorption provide negligible contributions to the 328–330-nm absorbance changes observed during aldehyde reduction in the presence of 20 mM pyrazole. The maximum slow phase amplitude contribution provided by pyrazole binding corresponds to 5–6% of the total absorbance change. Additional slow phase absorbance changes persisting at saturating concentrations of coenzyme and substrate reflect incomplete accumulation of the enzymic intermediate formed through hydride transfer in the rapid reaction phase. This intermediate contains no detectable amounts (less than 2%) of NADH, but exhibits spectral properties indistinguishable from those of enzyme · NAD⁺ · alcohol complexes examined.

4. It has been concluded in recent publications that an ordered ternary-complex mechanism involving equivalent sites is insufficient to account for the transient-state kinetics of liver alcohol dehydrogenase catalysis. These conclusions are shown to be based on an inadequate interpretation of the observations made. Results reported up to date provide no evidence whatsoever for the kinetic significance of subunit interactions leading to a non-equivalence of the enzymic sites. Subunit interactions resulting in a 'half-site' reactivity of the enzyme are definitely not at hand.

The transient-state kinetics of liver alcohol dehydrogenase catalysis have been extensively studied with a variety of alcohol and aldehyde substrates [1]. These studies have established that the enzymic oxidation of alcohols is governed by a single transient over the time scale accessible for observation by stop-flow techniques, whereas aldehyde reduction may exhibit a monophasic or biphasic time course depending on substrate structure. The observation that the enzymic reduction of aromatic aldehydes is associated with biphasic absorbance changes led Bernhard et al. [2] to propose that the two structurally identical subunits in liver alcohol dehydrogenase become kinetically non-equivalent during catalysis, such that the enzyme shows an apparent 'half-site' reactivity. This proposal was the subject of much debate until a theoretical analysis presented by Pettersson [3] indicated that the transient rate behaviour of the enzyme conforms well to that expected for an ordered ternary-complex mechanism involving catalytically equivalent sites.

The Pettersson theory provided analytical relationships for the dependence on substrate and coenzyme concentration of transients governing the enzymic reduction of aldehydes. Experimental tests reported by Kvassman and Pettersson [4]

established that the magnitude and saturation behaviour of rates and amplitudes for transients observed during acetaldehyde and benzaldehyde reduction was in reasonable quantitative agreement with the theoretical predictions. In particular, their measurements confirmed that the saturation value of the slow phase amplitude for benzaldehyde reduction by NADH is considerably smaller (about 15% of the total absorbance change) than the minimum value (50%) expected according to the 'half-site' reactivity hypothesis. Since it was recognized that both kinetic [3] and spectral [4] factors could account for the residual magnitude of this slow phase amplitude, Kvassman and Pettersson concluded that there is no evidence for any kinetically significant subunit non-equivalence in the liver alcohol dehydrogenase system.

Recently, however, Dunn et al. [5] published a paper stated to constitute a rebuttal in response to the inferences drawn by Kvassman and Pettersson. Using the pyrazole catalytic-suicide method of McFarland and Bernhard [6], Dunn et al. determined amplitudes for transients governing the enzymic reduction of a series of *para*-substituted benzaldehyde derivatives. Attempts were made to correct the data for absorbance changes due to pyrazole binding. The results thus obtained were claimed to be in substantial quantitative and qualitative disagreement with the ordered pathway proposed by Kvassman and Pettersson [4]. The conclusion was reiterated that more complicated mechanisms, most likely involving subunit interactions, must be considered. Spectral

Abbreviation. NAD²H, (4*R*)-4-deuterionicotinamide adenine dinucleotide.

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

evidence leading to the same conclusion was presented in a subsequent paper by Koerber and Dunn [7]. Arguments put forward by Dunn et al. [5] have been discussed also by Anderson and Dahlquist [8], who concluded that the biphasic rate behaviour of liver alcohol dehydrogenase reflects either a 'half-sites' reactivity mechanism or a complex and as yet not fully understood chemical mechanism.

The latter three reports make it clear that interpretation of the transient-state kinetics of liver alcohol dehydrogenase catalysis is still a matter of controversy. In our opinion, arguments put forward by Dunn et al. [5] should be given serious consideration in one particular respect. Kvassman and Pettersson [4], like previous investigators of the enzymic process of aldehyde reduction, based their interpretation on the simplifying assumption that absorbance changes observed at wavelengths around 328 nm reflect mainly conversion of enzyme-bound NADH into enzyme-bound NAD⁺. Their treatment did not account explicitly for absorbance changes possibly contributed by aldehyde and pyrazole binding, alcohol desorption, or by deviations from ideal single-turn-over conditions. As was pointed out by Kvassman and Pettersson [4] and by Dunn et al. [5], this leaves some uncertainty as to detailed origin of the slow phase absorbance changes persisting under conditions of substrate and co-enzyme saturation.

The present investigation was undertaken to eliminate the latter uncertainty and hence to settle once and for all the controversy concerning the kinetic equivalence of the liver alcohol dehydrogenase subunits. We have now derived relationships which clarify the implications of the Pettersson theory [3] with respect to the precise magnitude of amplitudes for transients observed in catalytic-suicide experiments. Results reported in the present paper provide the spectral and kinetic information required for application of these relationships to reactions now examined and to those considered by Dunn et al. [5] and by Koerber and Dunn [7]. It is shown that, adequately interpreted, data presented in the latter two reports confirm the general validity of the inferences drawn by Kvassman and Pettersson [4].

EXPERIMENTAL PROCEDURE

Material

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [2]. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [9] and are reported throughout as active-site concentrations.

The coenzymes NAD⁺ and NADH (Sigma Chemical Corp., grade III quality) were purified as described by Gurr et al. [10]. Pyrazole (Fluka AG) was recrystallized twice from water prior to use. Aldehydes and alcohols examined were obtained from Fluka AG (Buchs) and either recrystallized from water or distilled at reduced pressure under nitrogen before use. Other reagents were of highest available quality and used without further purification.

All experiments reported in the present paper were carried out at 25°C in 0.05 M pyrophosphate buffer (pH 8.75), prepared using water twice distilled from a quartz apparatus.

Kinetic Measurements

The kinetics of liver alcohol dehydrogenase catalysis of aldehyde reduction by NADH were examined and analyzed

using the equipment and general methods described previously [4,11]. Steady-state kinetic parameters for the enzymic (1 μM) reduction of *p*-nitrobenzaldehyde (10–100 μM) by a saturating concentration of NADH (100 μM) were determined by standard spectrophotometric methods [12]. Deviations from Michaelis-Menten kinetics indicative of substrate inhibition were observed at *p*-nitrobenzaldehyde concentrations above 100 μM.

The transient-state kinetics of the enzymic reduction of different benzaldehyde derivatives in the presence of 20 mM pyrazole were examined by stop-flow techniques at 328 nm, using 0.1 mM NADH and about 10 μM enzyme (concentrations indicated here and elsewhere in the paper refer to concentrations after mixing). Enzyme and coenzyme were preincubated prior to mixing with substrate and pyrazole. Rates and amplitudes of the transients observed were determined by regression analysis, as were the corresponding transient-state kinetic parameter values [4].

Spectral Measurements

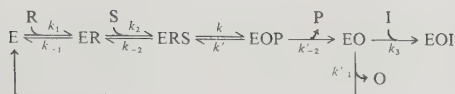
Absorption spectra in the 300–500-nm region were recorded on a Beckman Acta III spectrophotometer, instrumental settings being chosen to mimic as far as possible optical conditions of the stop-flow kinetic measurements. Absorption spectra for enzymic complexes were determined by standard absorption difference techniques, using 1-cm-pathlength split-compartment cuvettes and 30–50 μM enzyme. One and the same enzyme preparation was used for all experiments reported. Absorbance measurements in equilibrium solutions containing enzyme and NAD⁺ were performed within less than 1 min to avoid interferences from a slow blank reaction resulting (probably) in formation of NADH. Difference absorption data obtained under equilibrium conditions at 328 nm were checked and confirmed by stop-flow kinetic amplitude determinations for the absorbance changes associated with formation of the respective complexes.

RESULTS

Kinetic Theory

Let us consider an enzyme (E) catalyzing the conversion of two substrates (R, S) into two products (O, P) by an ordered ternary-complex mechanism with R as the leading substrate and O as the last-leaving product. When the enzyme is reacted with R and S in the presence of an inhibitor (I) which binds irreversibly to the catalytically produced complex EO, there will be a kinetic partitioning of this complex as indicated in Scheme 1. A predictable molar fraction $\{k_3[I]/(k_{-1} + k_3[I])\}$ of the complex will be withdrawn from further reaction through formation of the inactive species EOI. The remaining fraction $\{k_{-1}/(k_{-1} + k_3[I])\}$ will undergo dissociation to form a corresponding amount of free enzyme. The regenerated enzyme may then participate in a second catalytic cycle leading to formation and a second partitioning of species EO, and so forth. Assuming that there is no kinetically significant accumulation of products O and P during reaction, the molar fraction of complex EO undergoing dissociation will remain constant in each cycle. Under such conditions, the relationship

$$n = \sum_{i=0}^{\infty} \left(\frac{k_{-1}}{k_{-1} + k_3[I]} \right)^i = 1 + \frac{k_{-1}}{k_3[I]} \quad (1)$$



Scheme 1. Kinetic scheme for a catalytic-suicide reaction in enzyme systems operating by an ordered ternary-complex mechanism. E, R, S, O, and P denote enzyme, the two substrates, and the two products, respectively. I stands for an inhibitor which combines irreversibly to the EO complex with formation of a catalytically inactive species EOI



Scheme 2. Single-turnover mechanism considered in the present work. Notations as in Scheme 1. When applied to liver alcohol dehydrogenase reactions, R and O denote NADH and NAD⁺, respectively, while S stands for an aldehyde substrate, P for the corresponding alcohol product, and I for the catalytic-suicide inhibitor pyrazole

may be used to calculate the total number (n) of active-site equivalents of R (or S) which have been catalytically consumed when the reaction is completed, i.e. when the enzyme is entirely in the form of species EOI.

When $k_3[I] \gg k_{-1}, k'_{-2}$ we have $n \approx 1$ and the catalytic reaction will be limited essentially to a single turnover terminating with a rapid conversion of species EO into species EOI. It is then kinetically justified to reduce Scheme 1 as indicated in Scheme 2. The kinetic differential equations for the reaction in Scheme 2 have been solved by Pettersson [3] for the case that substrates are present in large excess to enzyme ($c_E \ll c_R, c_S$; c denotes the total concentration of the respective reactant). The solution prescribes that the time course of the reaction is governed by four exponential transients with predictable rates and amplitudes. Assuming that only two of the four transients are slow enough to be detected, approximate solutions relating to species EOP and EOI in Scheme 2 were shown to be given by

$$[EOI] = c_E \cdot (1 - e^{-r_1 t}) \quad (2)$$

$$[EOP] = c_E \cdot (e^{-r_1 t} - e^{-r_2 t}) \cdot F_1 \quad (3)$$

$$F_1 = \frac{r_1}{k'_{-2}} \quad (4)$$

where r_1 and r_2 denote rate parameters for the two detectable transients ($r_2 \gg r_1$). The corresponding relationship for species ERS can be similarly derived, which gives

$$[ERS] = c_E F_2 \cdot e^{-r_2 t} \quad (5)$$

$$F_2 = F_1 \cdot \frac{r_2 - k' - k'_{-2}}{k} \quad (6)$$

over the period of time where only the two slowest transients are of kinetic significance. If the additional assumption is made that R is present in saturating concentrations, the concentration of free enzyme may be neglected and we have

$$[ER] = c_E - [ERS] - [EOP] - [EOI] \quad (7)$$

The absorbance contribution (A) from enzymic species will then be given by

$$A = \varepsilon_{ER}[ER] + \varepsilon_{ERS}[ERS] + \varepsilon_{EOP}[EOP] + \varepsilon_{EOI}[EOI] \quad (8)$$

where ε denotes the absorption coefficient of the respective species. Insertion of Eqns (2–7) shows that Eqn (8) may be written as

$$A = (\varepsilon_{EOI} + A_1 \cdot e^{-r_1 t} + A_2 \cdot e^{-r_2 t}) \cdot c_E \quad (9)$$

where molar amplitudes of the two exponential transients are given by

$$A_1 = (1 - F_1)(\varepsilon_{ER} - \varepsilon_{EOI}) + F_1(\varepsilon_{EOP} - \varepsilon_{EOI}) \quad (10)$$

$$A_2 = F_1(\varepsilon_{ER} - \varepsilon_{EOP}) - F_2(\varepsilon_{ER} - \varepsilon_{ERS}). \quad (11)$$

It follows from Eqns (10) and (11) that

$$A_1 + A_2 = (1 - F_2)(\varepsilon_{ER} - \varepsilon_{EOI}) + F_2(\varepsilon_{ERS} - \varepsilon_{EOI}) \quad (12)$$

which reduces to

$$A_1 + A_2 = \varepsilon_{ER} - \varepsilon_{EOI} \quad (13)$$

when $\varepsilon_{ER} = \varepsilon_{ERS}$.

Detailed relationships for the dependence of r_1 and r_2 (and hence of F_1 and F_2) on c_R , c_S , and rate constants in Scheme 2 were derived by Pettersson [3]. According to these relationships we have

$$F_1 = \frac{c_S}{c_S + \phi_2 r_{1,\infty}} \cdot \frac{r_{1,\infty}}{k'_{-2}} \quad (14)$$

$$F_2 = \frac{c_S}{c_S + K_{app}} \left(1 - \frac{k' + k'_{-2}}{r_2} \right) \quad (15)$$

for saturating values of c_R , where $K_{app}(\phi_2)$ represents the transient-state (steady-state) kinetic parameter previously defined and shown to regulate the effect of c_S on r_2 (the steady-state reaction rate). Pettersson used the subscript ∞ to denote parameter values referring to saturating concentrations of both R and S, and showed that

$$r_{2,\infty} = k + k' + k'_{-2} \quad (16)$$

$$r_{1,\infty} = \frac{kk'_{-2}}{r_{2,\infty}} \quad (17)$$

It follows from Eqns (14–17) that saturation values of the parameters F_1 and F_2 are equal and given by

$$F_{1,\infty} = F_{2,\infty} = \frac{k}{k + k' + k'_{-2}} \quad (18)$$

which can be alternatively written as

$$F_{1,\infty} = F_{2,\infty} = 1 - \frac{k' + k'_{-2}}{r_{2,\infty}} \quad (19)$$

Absorption of Non-enzymic Reactants and Stable Enzymic Complexes

Standard spectrophotometric methods were used to determine absorption spectra for non-enzymic reactants and stable liver alcohol dehydrogenase complexes relevant to the present work. Pyrazole showed no significant ($\varepsilon < 1 \text{ M}^{-1} \text{ cm}^{-1}$) absorption at wavelengths above 300 nm. Absorption differences between aldehydes now considered and the corresponding alcohols were found to be less than $100 \text{ M}^{-1} \text{ cm}^{-1}$ above 325 nm. Absorption coefficients at 328 nm of coenzymes and enzymic species examined are given in Table 1. Estimates obtained by us agree closely with those reported in previous spectral studies of the enzyme system [13–16].

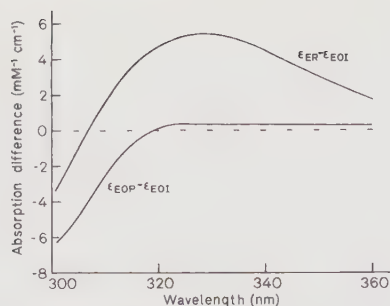


Fig. 1. Absorption differences in the 300–600-nm region between stable liver alcohol dehydrogenase complexes. Enzyme $\cdot$ NAD $^{+}$ $\cdot$ trifluoroethanol minus enzyme $\cdot$ NAD $^{+}$ $\cdot$ pyrazole ($\epsilon_{EOP} - \epsilon_{EOI}$) and enzyme $\cdot$ NADH minus enzyme $\cdot$ NAD $^{+}$ $\cdot$ pyrazole ($\epsilon_{ER} - \epsilon_{EOI}$) absorption difference spectra according to measurements made at 25°C in 50 mM pyrophosphate buffer, pH 8.75

In particular, our measurements confirm the observation of Hadorn et al. [14] that pyrazole binding to the enzyme $\cdot$ NAD $^{+}$ complex results in a small decrease in absorbance at 328 nm. Furthermore, data now obtained with trifluoroethanol and 2,2-dichloroethanol as the ligands lend support to previous conclusions that enzyme $\cdot$ NAD $^{+}$ $\cdot$ alcohol complexes exhibit essentially the same absorption coefficients as the enzyme $\cdot$ NAD $^{+}$ complex at this wavelength [5,8,14,16]. The enzyme $\cdot$ NAD $^{+}$ $\cdot$ trifluoroethanol minus enzyme $\cdot$ NAD $^{+}$ $\cdot$ pyrazole absorption difference spectrum recorded at 300–360 nm is given in Fig. 1 and will be assumed to be typical for absorbance changes associated with pyrazole binding to enzyme $\cdot$ NAD $^{+}$ $\cdot$ alcohol complexes in general. The enzyme $\cdot$ NADH minus enzyme $\cdot$ NAD $^{+}$ $\cdot$ pyrazole difference spectrum is included in Fig. 1. Table 2 lists the 328-nm absorption difference estimates required for detailed interpretation of amplitude data considered in this investigation.

Catalytic-Suicide Experiments

The transient-state kinetics of liver alcohol dehydrogenase catalysis were examined at 328 nm by the catalytic-suicide method of McFarland and Bernhard [6]. Reactions were performed at pH 8.75 in the presence of 20 mM pyrazole, using saturating concentrations of NADH [1,17] and limiting amounts of enzyme. Amplitudes (A_1, A_2) and apparent first-order rate constants (r_1, r_2 ; $r_1 \ll r_2$) for the two exponential transients governing the enzymic reduction of varied concentrations of benzaldehyde, *p*-methoxybenzaldehyde, *p*-chlorobenzaldehyde, and *p*-nitrobenzaldehyde were determined as detailed elsewhere [4]. The results confirmed previous reports that there is no detectable effect of substrate concentration on the total absorbance change occurring during reaction. Irrespective of the substrate and substrate concentration used, estimates of the sum $A_1 + A_2$ were found to differ insignificantly from the value 5400 M $^{-1}$ cm $^{-1}$ representing the absorption difference between the enzyme $\cdot$ NADH and enzyme $\cdot$ NAD $^{+}$ $\cdot$ pyrazole complex (Table 2).

Kinetic parameter values referring to saturating concentrations of substrate (and coenzyme) were obtained by regression analysis [4] and the results are given in Table 3. Estimates of rate parameters ($r_{2,\infty}$ and K_{app}) for the rapid

Table 1. Absorption data for coenzymes and stable liver alcohol dehydrogenase complexes

Absorption coefficients of enzymic species were calculated with the assumption that the enzyme contains two equivalent sites and refer to the active-site concentration of enzyme

Species	Notation in Scheme 2	Absorption coefficient at 328 nm
		M $^{-1}$ cm $^{-1}$
NADH	R	5600
NAD $^{+}$	O	80
Enzyme	E	400
Enzyme $\cdot$ NADH	ER	5800
Enzyme $\cdot$ NAD $^{+}$	EO	800
Enzyme $\cdot$ NAD $^{+}$ $\cdot$ pyrazole	EOI	450
Enzyme $\cdot$ NAD $^{+}$ $\cdot$ dichloroethanol	EOP	800
Enzyme $\cdot$ NAD $^{+}$ $\cdot$ trifluoroethanol	EOP	800

Table 2. Absorption difference estimates used for interpretation of transient-state kinetic data in the liver alcohol dehydrogenase system. Notations as in Table 1 and Scheme 2

Absorption difference	Estimate at 328 nm
	M $^{-1}$ cm $^{-1}$
$\epsilon_R - \epsilon_O$	5500 ($\pm$ 200)
$\epsilon_{ER} - \epsilon_{EOI}$	5400 ($\pm$ 300)
$\epsilon_{EOP} - \epsilon_{EOI}$	350 ($\pm$ 100)

one of the two transients agree well with those reported by Jacobs et al. [18].

Saturation values of kinetic parameters for the rapid transient governing the enzymic reduction of *p*-nitrobenzaldehyde could not be reliably determined due to the high rate of the transient and the low solubility of the substrate. Regression analysis of data obtained for the slow transient gave $r_{1,\infty} = 0.6$ s $^{-1}$ with half-saturation at 20 ($\pm$ 3) μ M *p*-nitrobenzaldehyde, consistent with the K_m value of 18 ($\pm$ 4) μ M determined by examination of the steady-state rate of reduction of the substrate by saturating concentrations of NADH. The amplitude values (A_1) determined for the slow transient at different concentrations of *p*-nitrobenzaldehyde are given in Fig. 2. Analysis of the latter data by the extrapolation method described previously [4] (the total absorbance change was assumed to correspond to $A_1 + A_2 = 5400$ M $^{-1}$ cm $^{-1}$) confirmed that the saturation behaviour of A_1 conforms to an apparent substrate dissociation equilibrium constant of 20 μ M and gave $A_{1,\infty} = 300$ ($\pm$ 200) M $^{-1}$ cm $^{-1}$.

Absorption of Enzyme $\cdot$ NADH $\cdot$ Aldehyde Complexes

The present and previously reported kinetic data for reactions considered in Table 3 [4,5,18,19] indicate that $k' + k'_2 \ll r_2$ over the range of substrate concentrations (c_S) now examined. This means, according to Eqn (15), that the magnitude of the kinetic parameter F_2 will be determined mainly by the magnitude of c_S relative to that of K_{app} (the apparent equilibrium constant for aldehyde binding to the enzyme $\cdot$ NADH complex [4,18]). Aldehyde concentrations used in the present and previously reported kinetic studies

Table 3. Transient-state kinetic parameter values for the enzymic reduction of aromatic aldehydes by NADH
Estimates obtained by regression analysis of kinetic data recorded at 25 °C in 50 mM pyrophosphate buffer, pH 8.75

Substrate	Concn range μM	K_{app}	$r_{2,\infty}$ s ⁻¹	$r_{1,\infty}$	$A_{1,\infty}$ M ⁻¹ cm ⁻¹
<i>p</i> -Methoxybenzaldehyde	50–5000	350 (± 50)	190 (± 40)	8 (± 2)	1200 (± 200)
Benzaldehyde	50–5000	400 (± 70)	320 (± 70)	10 (± 2)	800 (± 100)
<i>p</i> -Chlorobenzaldehyde	50–200	60 (± 10)	380 (± 100)	1.6 (± 0.3)	600 (± 200)
<i>p</i> -Nitrobenzaldehyde	50–150	—	> 700	0.6 (± 0.1)	300 (± 200)

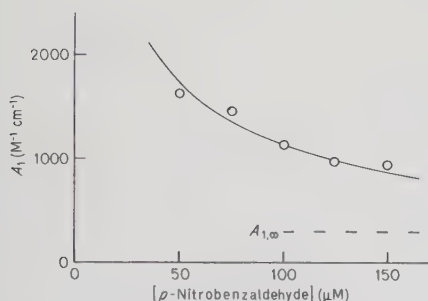


Fig. 2. Dependence on substrate concentration of the slow phase amplitude for the enzymic reduction of *p*-nitrobenzaldehyde by NADH. Amplitudes (A_1) recorded at 328 nm on reacting liver alcohol dehydrogenase (9.2 μM) and NADH (100 μM) with varied concentrations of substrate at 25 °C in 50 mM pyrophosphate buffer (pH 8.75) containing 20 mM pyrazole. The curve drawn was calculated from Eqns (4), (10), and (14) using $\epsilon_{ER} - \epsilon_{EO} = 5400 \text{ M}^{-1} \text{ cm}^{-1}$, $\phi_2 r_{1,\infty} = 20 \mu\text{M}$, and $A_{1,\infty} = 300 \text{ M}^{-1} \text{ cm}^{-1}$

range from values much lower than K_{app} to values much higher than K_{app} (cf. Table 3), i.e. the corresponding values of F_2 vary from close to zero to close to unity. According to Eqn (12), therefore, the absence of detectable effects of substrate concentration on the total absorbance change $A_1 + A_2$ establishes that there is no kinetically significant difference between the absorption coefficients ϵ_{ER} and ϵ_{ERS} at the wavelengths examined. Confirmatively, the observed magnitude of $A_1 + A_2$ ($5400 \text{ M}^{-1} \text{ cm}^{-1}$) agrees with that predicted by Eqn (13) and the spectral data in Table 2.

Deviations from Single-Turnover Conditions

The observation that Eqn (13) applies within experimental precision (about $\pm 200 \text{ M}^{-1} \text{ cm}^{-1}$) provides the additional information that there are no detectable deviations from ideal single-turnover conditions in the above catalytic-suicide experiments. Such deviations would be expected to result in additional consumption of free NADH (R) with formation of NAD⁺ (O) at a rate comparable to that of the slow exponential transient. Defining n as in Eqn (1), the corresponding contribution to the total absorbance change during reaction should be given by $(n-1)(\epsilon_R - \epsilon_O)$. Since $\epsilon_R - \epsilon_O = 5500 \text{ M}^{-1} \text{ cm}^{-1}$ (Table 2), the present results indicate that $n < 1.036$.

Assuming that $k'_1 = 12 \text{ s}^{-1}$ [4, 20], it then follows from Eqn (1) that $k_3[I] > 330 \text{ s}^{-1}$ with 20 mM pyrazole (I) at pH 8.75. This is in conflict with the report of McFarland and Bernhard [6] that $k_3[I] = 105 \text{ s}^{-1}$ (corresponding to $n = 1.11$)

under such conditions. According to the pyrazole binding data of Andersson et al. [11], however, we have $k_3[I] = 460 \text{ s}^{-1}$ ($n = 1.026$) with 20 mM pyrazole at pH 8.75. The present results lend support to the latter report and establish that the presence of 20 mM pyrazole provides a satisfactorily close approach to ideal ($n = 1$) single-turnover conditions in standard catalytic-suicide experiments.

Judging from the reported pH dependence of k'_1 [17, 21] and k_3 [11], the same should hold true independently of pH within the pH range 5–10. The report of McFarland and Chu [22] that $k_3[I] = 102 \text{ s}^{-1}$ with 20 mM pyrazole at pH 7 cannot be trusted; it would correspond to $n > 2$ and is inconsistent with the magnitude of the total absorbance changes observed during aldehyde reduction at pH 7 [22, 23], as well as with the pyrazole association rates ($k_3[I] = 5600 \text{ s}^{-1}$) observed by Andersson et al. [11].

Amplitude Values Calculated from Eqn (10)

Data in Table 2 provide the spectral information required for interpretation of the observed saturation values of A_1 in terms of Eqn (10). We will now consider the effect on the magnitude of $A_{1,\infty}$ of the kinetic parameter $F_{1,\infty}$ with respect to reactions considered in the present investigation.

The magnitude of $F_{1,\infty}$ for benzaldehyde reduction by NADH was calculated from Eqn (18) using the rate constant values reported by Kvassman and Pettersson [24]. The magnitude of $F_{1,\infty}$ for the corresponding reaction with NAD²H as the coenzyme was similarly estimated with the assumption that rate constants for the hydride transfer step (k and k') are subject to an isotope effect of 2.5 [18]. Estimates of $F_{1,\infty}$ for other reactions in Table 3 were obtained from Eqn (19) using the standard approximations $k'_2 = r_{1,\infty}$ and $k' = r'_{1,\infty}$ [3, 24]. Values of $r_{1,\infty}$ and $r_{2,\infty}$ were taken from Table 3 and values of $r'_{1,\infty}$ from the report of Hardman et al. [19]. The magnitude of $F_{1,\infty}$ for *p*-methoxybenzaldehyde reduction by NAD²H was similarly determined using $r_{1,\infty} = 5 \text{ s}^{-1}$ [5], $r_{2,\infty} = 60 \text{ s}^{-1}$ [5], and $r'_{1,\infty} = 8.5 \text{ s}^{-1}$ [19]. Values of $F_{1,\infty}$ for *p*-nitrobenzaldehyde reduction were estimated using $r'_{1,\infty} < 1$ [19], $r_{1,\infty} < 0.7 \text{ s}^{-1}$, and $r_{2,\infty} > 200 \text{ s}^{-1}$ [5, 7] (cf. Table 3).

Table 4 shows the $F_{1,\infty}$ values thus obtained. The corresponding values of $A_{1,\infty}$, calculated from Eqn (10) using the spectral data in Table 2, are included in Table 4.

DISCUSSION

Applicability of Relationships Derived for Scheme 2

The pyrazole catalytic-suicide method of McFarland and Bernhard [6] has been extensively used for characterization of the transient-state rate behaviour of liver alcohol dehydrogenase under pseudo-single-turnover conditions. Kvassman

Table 4. Estimates of the kinetic parameter $F_{1,\infty}$ for reactions catalyzed by liver alcohol dehydrogenase. The corresponding saturation values ($A_{1,\infty}$) of the slow phase amplitude were calculated from Eqn (10) using the spectral data in Table 2

Substrate	Coenzyme	$F_{1,\infty}$	$A_{1,\infty}$ $M^{-1} cm^{-1}$
<i>p</i> -Methoxybenzaldehyde	NADH	0.85	1100
Benzaldehyde	NADH	0.91	800
<i>p</i> -Chlorobenzaldehyde	NADH	0.97	500
<i>p</i> -Nitrobenzaldehyde	NADH	≈ 1.00	≈ 400
<i>p</i> -Methoxybenzaldehyde	NAD ² H	0.78	1500
Benzaldehyde	NAD ² H	0.86	1100
<i>p</i> -Nitrobenzaldehyde	NAD ² H	≈ 1.00	≈ 400

and Pettersson [4] analyzed such data in terms of relationships derived for the ordered mechanism in Scheme 2 [3], but no attempt was made to apply the kinetic theory for detailed interpretation of the slow phase absorbance changes (about $800 M^{-1} cm^{-1}$ for benzaldehyde reduction by NADH) persisting under conditions of substrate and coenzyme saturation. It appeared from the available pyrazole association rate data [6, 22] that enzyme recycling might provide a major contribution (about $600 M^{-1} cm^{-1}$) to these absorbance changes, which would make it of minor interest to estimate and analyze the small contribution provided by the actual transient.

It seems likely, however, that the preliminary pyrazole association rate data of McFarland and Bernhard [6] and MacFarland and Chu [22] may have been affected by the presence of trace amounts of NADH [25]. According to the more extensive binding studies performed by Andersson et al. [11], 20 mM pyrazole would be expected to render enzyme recycling kinetically insignificant in catalytic-suicide experiments. The present results confirm this expectation, which justifies application of the Pettersson theory [3] for detailed quantitative analysis of amplitudes (A_1 and A_2) of the two transients governing the enzymic reduction of aromatic aldehydes. Considering the spectral data now reported, it seems reasonable to assume that non-enzymic reactants contribute negligibly to the magnitude of amplitudes recorded at 328–330 nm. Eqns (10) and (11), therefore, can be reliably used to test the predictions of Scheme 2 as concerns amplitudes determined at these wavelengths with saturating concentrations of NADH and limiting amounts of enzyme.

With respect to such tests, it is of primary interest to note that variation of the substrate concentration has no detectable effect on the total absorbance change ($A_1 + A_2$) occurring during reactions examined. The implication of this observation, which is consistent with Eqns (10) and (11) and establishes that absorbance changes associated with aldehyde binding to the enzyme · NADH complex are kinetically insignificant, is that it suffices to account for the magnitude of one, only, of the two amplitudes A_1 and A_2 . We prefer to discuss the slow phase amplitude A_1 , the magnitude of which can be reliably determined with any substrate and for any concentration of the substrate.

Spectral Parameters Affecting the Magnitude of A_1

Eqn (10) shows that the slow phase amplitude A_1 is dependent on two absorption differences, one reflecting conversion of the enzyme · NADH complex into the enzyme

· NAD⁺ · pyrazole complex ($\epsilon_{ER} - \epsilon_{EOI}$) and one reflecting pyrazole binding to the enzyme · NAD⁺ · alcohol complex ($\epsilon_{EOP} - \epsilon_{EOI}$). The former absorption difference can be estimated by conventional absorption spectrophotometry or from the total absorbance change ($A_1 + A_2$) occurring during catalytic-suicide reactions performed with saturating concentrations of coenzyme. The value ($5400 M^{-1} cm^{-1}$) thus determined in the present investigation agrees well with previously reported estimates of $\epsilon_{ER} - \epsilon_{EOI}$ or $A_1 + A_2$ at 328 nm. According to Fig. 1, $\epsilon_{ER} - \epsilon_{EOI}$ should exhibit approximately the same magnitude at the wavelength (330 nm) where Dunn et al. [5] performed their kinetic measurements. Confirmatively, total absorbance changes observed by Dunn et al. for the enzymic reduction of benzaldehyde and *p*-methoxybenzaldehyde correspond well to $5400 M^{-1} cm^{-1}$. Their estimates of $A_1 + A_2$ for *p*-chlorobenzaldehyde and *p*-nitrobenzaldehyde are slightly lower ($4300 - 4400 M^{-1} cm^{-1}$), but this seems to reflect a loss of experimental precision introduced by the large dead-time corrections which have to be applied in amplitude determinations with the latter substrates. There is no reason to believe that the total absorbance change should be significantly different with different substrates, at least not at wavelengths around 330 nm where absorption differences between aldehyde substrates examined and the corresponding alcohol products are of negligible magnitude.

We have not made any attempts to obtain direct estimates of the absorption difference ($\epsilon_{EOP} - \epsilon_{EOI}$) reflecting pyrazole binding to the catalytically productive enzyme · NAD⁺ · alcohol complexes formed during reactions considered in Table 3. As pointed out by Dunn et al. [5], there is strong reason to believe that alcohol dissociation from the productive ternary complexes does not lead to any significant absorbance changes at 328–330 nm [7, 14, 16]. This means that absorbance changes associated with pyrazole binding to such complexes are likely to derive mainly from the step of pyrazole binding to the enzyme · NAD⁺ complex. The present results are fully consistent with this idea. It, therefore, seems justified to interpret A_1 values for all reactions considered using one and the same estimate of $\epsilon_{EOP} - \epsilon_{EOI}$. The estimate now obtained using trifluoroethanol or dichloroethanol as the ligand P ($350 M^{-1} cm^{-1}$) agrees well with that ($255 M^{-1} cm^{-1}$) determined for $\epsilon_{EO} - \epsilon_{EOI}$ at 328 nm by Hadorn et al. [14]. According to Fig. 1, $\epsilon_{EOP} - \epsilon_{EOI}$ can be safely assumed to exhibit the same magnitude at 330 nm as at 328 nm.

Kinetic Parameters Affecting the Magnitude of A_1

Eqn (10) expresses the substrate concentration dependence of A_1 in terms of a kinetic parameter F_1 which is proportional to the rate parameter r_1 for the slow transient. It follows from Eqn (14) that the magnitude of F_1 should increase with increasing substrate concentration (c_s) towards the saturation value $F_{1,\infty}$ given by Eqn (18), half-saturation being obtained for $c_s = \phi_2 r_{1,\infty}$. The saturation behaviour of the slow phase rate and amplitude observed for benzaldehyde reduction by NADH has been previously shown to be consistent with these predictions of Scheme 2 [4]. Saturation effects observed by Dunn et al. [5], as well as the results in Fig. 2, can be analogously explained. The transient-state and steady-state kinetic data now reported provide consistent evidence that $\phi_2 r_{1,\infty} \approx 20 \mu M$ with *p*-nitrobenzaldehyde as the substrate.

There seems to be no disagreement about the mechanistic origin of the saturation behaviour of A_1 [4, 5]. It should suffice, therefore, to consider the predictions of Scheme 2 with respect to the saturation value $A_{1,\infty}$, which is dependent

on the precise magnitude of the kinetic parameter $F_{1,\infty}$. Methods used for calculation of the $F_{1,\infty}$ values in Table 4 are in accordance with the kinetic theory for Scheme 2 [3,24] and provide sufficiently precise estimates for the purpose of the present investigation. The results in Table 4 confirm the supposition of Dunn et al. [5] that the magnitude of $F_{1,\infty}$ is subjected to appreciable electronic substituent effects, such that $F_{1,\infty}$ becomes close to unity with *p*-nitrobenzaldehyde as the substrate.

Spectral Data Presented by Koerber and Dunn

Koerber and Dunn [7] examined the wavelength dependence of the rapid and slow phase absorbance changes occurring during the enzymic reduction of about 90 μM *p*-nitrobenzaldehyde. The absorbance change in each reaction phase was found to be characterized by a difference spectrum agreeing closely with that of enzyme-bound NADH at wavelengths above 320 nm. Hence the authors drew the sound inference that enzyme-bound NADH is oxidized not only in the rapid reaction phase, but also in the slow one. This was taken to indicate that the slow reaction step limiting the rate of formation of the enzyme $\cdot \text{NAD}^+$ $\cdot$ pyrazole complex (the conversion of EOP into EOI in Scheme 2) is closely coupled with NADH oxidation, as might be expected according to the half-site reactivity hypothesis. Koerber and Dunn concluded that ligand-mediated subunit interactions convey catalytic non-equivalence to the enzymic sites. The mechanism proposed by Kvassman and Pettersson [4] was stated not to provide a satisfactory description of the observations made.

The predictions of Scheme 2 in this respect are expressed by Eqns (10) and (11). It follows from these amplitude expressions that NADH oxidation always must be assumed to contribute to the absorbance changes occurring in both reaction phases, the precise magnitude of the contributions being regulated by the magnitude of the kinetic parameter F_1 . According to Eqn (14) and the kinetic data discussed in the preceding section, we should have $F_1 \approx 0.8$ with 90 μM *p*-nitrobenzaldehyde ($\phi_2 r_{1,\infty} \approx 20 \mu\text{M}$ and $F_{1,\infty} \approx 1$). For this value of F_1 , it follows from Eqn (10) that slow phase absorbance changes observed at wavelengths above 320 nm will include an 80% contribution from the small and featureless absorption difference reflecting pyrazole binding ($\epsilon_{\text{EOP}} - \epsilon_{\text{EOI}}$ in Fig. 1) and a 20% contribution from the large and characteristic absorption difference reflecting NADH oxidation ($\epsilon_{\text{ER}} - \epsilon_{\text{EOI}}$ in Fig. 1). The corresponding wavelength dependence of A_1 is shown in Fig. 3 and, in our opinion, provides an excellent description of the characteristic features of the difference spectra recorded by Koerber and Dunn [7]. The slow phase oxidation of enzyme-bound NADH observed by the latter authors is qualitatively consistent with the ordered mechanism in Scheme 2. The observed apparent amplitude contribution (about 25% of a total absorbance change of $5400 \text{ M}^{-1} \text{ cm}^{-1}$) from such slow phase NADH oxidation is quantitatively consistent with the predictions of Eqn (10), but considerably smaller than would be expected according to the half-sites reactivity hypothesis.

Amplitude Corrections Applied by Dunn et al.

When evaluating their kinetic data, Dunn et al. [5] attempted to consider the amplitude contributions provided by pyrazole binding. They concluded from the absorbance changes observed in a model reaction that formation of the enzyme $\cdot \text{NAD}^+$ $\cdot$ pyrazole complex in catalytic-suicide ex-

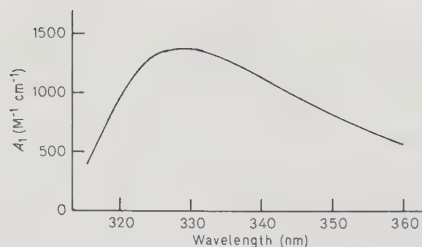


Fig. 3. Wavelength dependence predicted by Scheme 2 for the amplitude of the slow transient governing the enzymic reduction of about 90 μM *p*-nitrobenzaldehyde. Amplitude values (A_1) calculated from Eqn (10) for $F_1 = 0.8$ using the spectral data in Fig. 1

periments is associated with an absorption increase of about $780 \text{ M}^{-1} \text{ cm}^{-1}$ at 330 nm. This conclusion is in substantial disagreement with the present observation that pyrazole binding to enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complexes results in an absorption decrease of about $350 \text{ M}^{-1} \text{ cm}^{-1}$ at wavelengths around 330 nm (Fig. 1). Since the enzyme $\cdot \text{NAD}^+$ $\cdot$ pyrazole complex cannot possibly be formed from an enzyme species exhibiting negative absorbance, the conclusion of Dunn et al. would be difficult to reconcile also with the present (Table 1) and previous [14] observations that the absolute absorption of the latter ternary complex is lower than $780 \text{ M}^{-1} \text{ cm}^{-1}$.

It appears from the arguments put forward by Dunn et al. that they wished to determine the absorbance change contributed by pyrazole binding to the enzyme $\cdot \text{NAD}^+$ complex. In their model experiment, however, they measured the absorbance change associated with NADH displacement resulting from NAD^+ and pyrazole binding to the enzyme $\cdot \text{NADH}$ complex. We have confirmed the observation of Weidig et al. [16] that the isosbestic point for free and enzyme-bound NADH is at 325 nm in pyrophosphate buffer at pH 8.75. This means that the 300 nm absorbance increase in the model reaction examined by Dunn et al. is likely to derive mainly from NADH desorption from the enzyme. Their experimental observation does not appear to be inconsistent with the spectral data now reported.

Dunn et al. [5] argued that the absorbance change observed in the model reaction should be added as a correction term to the slow phase amplitude values obtained in their kinetic experiments. Such an amplitude correction is unsound for two main reasons. Firstly, the absorbance change associated with a certain reaction step cannot be taken to represent the amplitude contribution provided by this reaction step, but has to be weighted by an appropriate concentration-dependent kinetic factor [26] such as F_1 in Eqn (10). Secondly, the model reaction examined by Dunn et al. is irrelevant for the absorbance changes associated with pyrazole binding in catalytic-suicide reactions. Whether or not the enzymic sites are assumed to be kinetically equivalent, it is quite clear that such reactions do not involve any net displacement of NADH or binding of NAD^+ . Our discussion of amplitude data presented by Dunn et al., therefore, will be confined to the uncorrected experimental observations made.

Amplitude Data Presented by Dunn et al.

Data reported by Dunn et al. [5] for the enzymic reduction of *p*-nitrobenzaldehyde by NADH require particular

consideration. Estimates of A_1 for this reaction were observed to decrease approximately from $2400 \text{ M}^{-1} \text{ cm}^{-1}$ to $1500 \text{ M}^{-1} \text{ cm}^{-1}$ as the substrate concentration was raised from about $12 \mu\text{M}$ to $190 \mu\text{M}$. Hence the authors concluded that the slow phase amplitude is 'nearly independent of substrate concentration over the range investigated', and the corresponding saturation value of A_1 was stated to represent 50% of the total absorbance change (as might be expected according to the half-sites reactivity hypothesis). The latter datum is unreliable as an estimate of $A_{1,\infty}$ for several reasons. Firstly, it was obtained by application of the above unjustified amplitude correction. Secondly, it is based on a considerable underestimation of the total absorbance change occurring during reaction. Thirdly, the datum corresponds to an observed A_1 value of at least $1800 \text{ M}^{-1} \text{ cm}^{-1}$. Since this value is larger than that observed at the highest substrate concentration tested, it cannot possibly represent the saturation value of the slow phase amplitude. Finally, the *p*-nitrobenzaldehyde reduction experiments of Dunn et al. were performed using a coenzyme concentration ($15 \mu\text{M}$) which is less than 85% saturating (due to mutual depletion effects) and certainly not greatly exceeding the active-site concentration of enzyme ($12 \mu\text{M}$) in their experiments. Under such conditions, the kinetic differential equations for the reaction cannot be analytically solved with acceptable precision [26]. This means that absorbance changes observed cannot be adequately described in terms of exponential transients (apparent first-order kinetics are not at hand). A statistical fit of exponential transients to the experimental data may certainly provide rate and amplitude estimates, but these estimates cannot be reliably interpreted in terms of analytical relationships, at least not in terms of the relationships derived by Pettersson [3] and in the present paper.

The results in Fig. 2, on the other hand, were obtained using a coenzyme concentration ($100 \mu\text{M}$) which is more than 99% saturating and about 10-fold higher than the active-site concentration of enzyme. This difference in experimental conditions appears to explain why A_1 values observed by Dunn et al. for *p*-nitrobenzaldehyde concentrations above $50 \mu\text{M}$ are considerably higher than those reported in Fig. 2. The slow phase amplitude (about $1300 \text{ M}^{-1} \text{ cm}^{-1}$) observed by Koerber and Dunn [7] for the enzymic reduction of $89.7 \mu\text{M}$ *p*-nitrobenzaldehyde under apparent first-order conditions agrees excellently with data in Fig. 2. The latter data provide an estimate of $A_{1,\infty}$ corresponding to 6% of the total absorbance change.

Other reactions examined by Dunn et al. [5] seem to have been performed under such conditions that the A_1 values observed can be analytically interpreted. The corresponding estimates of $A_{1,\infty}$ are given in Table 5 (at a $100 \text{ M}^{-1} \text{ cm}^{-1}$ level of precision) and were obtained by subtracting $780 \text{ M}^{-1} \text{ cm}^{-1}$ from the 'corrected' A_1 values reported by Dunn et al. for the highest substrate concentrations tested. The $A_{1,\infty}$ value for benzaldehyde reduction by NADH was calculated as 15% [5] of a total absorbance change of $5400 \text{ M}^{-1} \text{ cm}^{-1}$. $A_{1,\infty}$ values now observed (Table 3) and calculated theoretically (Table 4) are included in Table 5 for comparison.

Dunn et al. [5] concluded that the kinetics of enzymic reduction of benzaldehyde and *para*-substituted benzaldehyde derivatives by NADH or NAD^2H are in substantial qualitative and quantitative disagreement with Scheme 2 as concerns the absolute magnitude and relative insensitivity to substituent and isotope effects of amplitudes of the transients governing such reactions. The results in Table 5 lend no support to this conclusion. The observed electronic substituent

Table 5. Experimentally observed saturation values $A_{1,\infty}$ for the slow phase amplitude, compared to those predicted by Scheme 2

Reaction	$A_{1,\infty}$ pre- dicted	$A_{1,\infty}$ observed	
		Dunn et al. [5]	this work
	$\text{M}^{-1} \text{ cm}^{-1}$		
NADH + <i>p</i> -methoxybenzaldehyde	1100	—	1200
NADH + benzaldehyde	800	800	800
NADH + <i>p</i> -chlorobenzaldehyde	500	600	600
NADH + <i>p</i> -nitrobenzaldehyde	400	—	300
NAD^2H + <i>p</i> -methoxybenzaldehyde	1500	1500	—
NAD^2H + benzaldehyde	1100	1100	—
NAD^2H + <i>p</i> -nitrobenzaldehyde	400	—	—

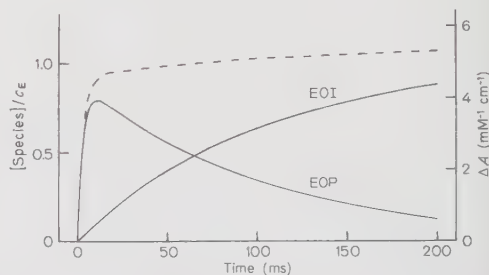


Fig. 4. Computer simulation of the enzymic reduction of benzaldehyde by NADH under conditions of substrate and coenzyme saturation. Full curves indicate transient concentration changes of species EOP and EOI in Scheme 2, calculated from Eqns (2–4) using previously reported [24] estimates of rate constants and kinetic parameters. The dashed curve shows the corresponding decrease in absorbance at 328 nm (A), calculated from Eqns (9–11) using data in Table 2 with the assumption that $\epsilon_{\text{ERS}} = \epsilon_{\text{ER}}$.

effects are in the right direction and of the comparatively low magnitude prescribed by Eqn (10). The same applies for the deuterium isotope effect, which can be detected with benzaldehyde and *p*-methoxybenzaldehyde as the substrate but would be expected to be negligibly small for *p*-nitrobenzaldehyde reduction; amplitude data presented by Koerber and Dunn [7] confirm the latter expectation.

The observed magnitude of $A_{1,\infty}$ actually agrees within $100 \text{ M}^{-1} \text{ cm}^{-1}$ with that calculated from Eqn (10) for each individual reaction considered in Table 5. In other words, Eqn (10) accounts for the observed saturation value of the slow phase absorbance change within 2% of the total absorbance change for all reactions examined. It may be concluded that experimental data summarized in Table 5 are in remarkably excellent agreement with the predictions of Scheme 2.

Mechanistic Implications

According to Scheme 2, the appearance of biphasic absorbance changes during the enzymic reduction of aromatic aldehydes in catalytic-suicide experiments can be attributed to accumulation of the intermediate EOP [4,27]. This is illustrated by the computer simulations in Fig. 4, which are

based on the present kinetic theory and refer to the reduction of saturating concentrations of benzaldehyde by saturating concentrations of NADH. The requisites for a kinetically detectable accumulation of species EOP were discussed by Pettersson [3], who pointed out that $A_{1,\infty}$ cannot be assumed to be of negligible magnitude unless the rate of breakdown of EOP is negligible compared to its rate of formation ($k \gg k' + k'_2$ or $F_{1,\infty} \approx 1$). Kvassman and Pettersson [4] suggested that the non-vanishing magnitude of $A_{1,\infty}$ for benzaldehyde reduction might reflect also minor absorption differences between the enzyme $\cdot$ NAD⁺ $\cdot$ alcohol complex (EOP) and the enzyme $\cdot$ NAD⁺ $\cdot$ pyrazole complex (EOI). The results in Table 5 establish that detailed consideration of the latter absorption difference and the actual magnitude of the kinetic parameter $F_{1,\infty}$ is sufficient to account for the observed $A_{1,\infty}$ values within experimental precision. Absorbance contributions from pyrazole binding to the saturation value of the slow phase amplitude are small and approximately constant (about 300 M⁻¹ cm⁻¹) for all reactions considered. Variations in the magnitude of $A_{1,\infty}$ derive mainly from differences in the magnitude of the kinetic parameter $F_{1,\infty}$.

The present results, therefore, eliminate the previous uncertainty concerning the detailed origin of the slow phase absorbance changes persisting under conditions of substrate and coenzyme saturation. Data now reported reaffirm the conclusion of Kvassman and Pettersson [4] that the transient-state kinetics of liver alcohol dehydrogenase are fully consistent with an ordered ternary-complex mechanism involving equivalent enzymic sites. Adequately interpreted, results presented by Dunn et al. [5] and Koerber and Dunn [7] confirm the general validity of this conclusion.

One may ask then whether or not results summarized in Table 5 are consistent also with the half-sites reactivity hypothesis [2]. To answer this question, attention should be drawn to the fact that the latter hypothesis relates the biphasic rate behaviour of liver alcohol dehydrogenase to a kinetic situation which is formally analogous to that in Scheme 2. Coenzyme and substrate binding are assumed to be followed under single-turnover conditions by two kinetically significant processes at the ternary-complex level. A rapid step of hydride transfer from NADH at one subunit is envisaged to precede slow NADH oxidation at the second subunit, the latter process most likely being rate-limited by alcohol desorption from the intermediate formed in the rapid hydride transfer step. The fundamental kinetic characteristics of such a mechanism are adequately expressed by Scheme 2 and may be discussed in view of the analytical relationships derived for this kinetic scheme. In other words, there seems to be no disagreement between proponents and opponents of the half-site reactivity hypothesis as to the kinetic situation which is at hand. The controversy concerns the nature, spectral properties, and mode of formation and breakdown of the intermediate [7] (EOP in Scheme 2) which accumulates during the enzymic reduction of aromatic aldehydes.

According to the half-sites reactivity hypothesis, this intermediate should represent a mixed-type ternary complex containing NAD⁺ at one subunit and unreacted NADH at the second subunit, i.e. the absorption of species EOP would be expected to be approximately the mean (about 3300 M⁻¹ cm⁻¹ at 328–330 nm) of that of the enzyme $\cdot$ NADH and enzyme $\cdot$ NAD⁺ complexes. The $A_{1,\infty}$ values calculated from Eqn (10) using the corresponding estimate of $\epsilon_{\text{EOP}} - \epsilon_{\text{EOI}}$ (2850 M⁻¹ cm⁻¹) range from 2850 M⁻¹ cm⁻¹ to 3400 M⁻¹ cm⁻¹ (53–62% of the total absorbance change) for reactions considered

in Table 5, and are obviously inconsistent with the observations made. The observed $A_{1,\infty}$ values vary from 300 M⁻¹ cm⁻¹ to 1400 M⁻¹ cm⁻¹ (6–26% of the total absorbance change) and correspond to $\epsilon_{\text{EOP}} - \epsilon_{\text{EOI}} = 390 (\pm 60) \text{ M}^{-1} \text{ cm}^{-1}$. The present results, therefore, provide clear evidence that the actual absorption of species EOP differs insignificantly (less than 2%) of the reduced form of the coenzyme are present in the ternary complex which accumulates during the enzymic reduction of aromatic aldehydes. The latter ternary complex exhibits absorption properties indistinguishable from those of enzyme $\cdot$ NAD⁺ $\cdot$ alcohol complexes now examined (Table 1).

These observations, which are supported by the spectral data presented by Koerber and Dunn [7], render the half-sites reactivity hypothesis completely untenable as a mechanistic explanation for the biphasic absorbance changes associated with aromatic aldehyde reduction in the liver alcohol dehydrogenase system. Ligand-mediated subunit interactions conveying a more or less pronounced catalytic non-equivalence to the active sites can certainly be anticipated to be at hand in all oligomeric enzymes. In liver alcohol dehydrogenase, however, the effects of such interactions are too small to be experimentally detected even at the high level of precision considered in the present investigation. The present results establish the validity of the inferences drawn by Kvassman and Pettersson [4] in the latter respect, as well. There is no evidence whatsoever for the kinetic significance of subunit interactions leading to a site non-equivalence in liver alcohol dehydrogenase, particularly not of subunit interactions resulting in a half-site reactivity of the enzyme.

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Effect of pH on Pyrazole Binding to Liver Alcohol Dehydrogenase

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1. Kinetic and equilibrium data have been determined at different pH between 4 and 10 for binding of the inhibitor pyrazole to liver alcohol dehydrogenase and to the binary complexes formed between enzyme and NADH or NAD⁺.

2. Pyrazole binding to free enzyme requires the protonated form of an ionizing group with a pK_a of 9.2, agreeing with the pK_a value reported for the water molecule bound at the catalytic zinc ion of the enzyme subunit. The rate of association of the inhibitor to the enzyme · NAD⁺ complex exhibits a similar pK_a -7.6-dependence attributable to ionization of zinc-bound water in the latter binary complex. These observations lend support to the idea that pyrazole combines to the catalytic zinc ion on complex formation with the enzyme, zinc-bound water most likely being displaced by the inhibitor.

3. The rate of dissociation of the inhibitor from the ternary enzyme · NAD⁺ · pyrazole complex is proportional to the hydrogen ion concentration over the examined pH range (4–8). This effect of pH, which is proposed to reflect ionization of the enzyme-bound inhibitor with a pK_a value below 4 (indirectly estimated to 2.4), accounts for the exceptional stability of the ternary complex at neutral and alkaline pH. It is concluded that pyrazole, by analogy to water and alcohol ligands, undergoes a drastic pK_a perturbation on binding to the catalytic zinc ion in the enzyme · NAD⁺ complex.

Pyrazole is an effective inhibitor of the liver alcohol dehydrogenase reaction, strictly competitive with the alcohol substrate [1]. Theorell et al. demonstrated that pyrazole inhibition is attributable to the formation of an exceptionally firm enzyme · NAD⁺ · pyrazole complex showing a characteristic difference absorption band at about 295 nm [2]. Due to the stability and chromophoric properties of this ternary complex, pyrazole has turned out to be a most useful ligand from a methodological point of view. Transient-state kinetic methods based on the absorbance changes associated with pyrazole binding have been applied to monitor formation of the enzyme · NAD⁺ complex during catalysis and to study the process of ternary-complex formation with non-chromophoric substrate and coenzyme analogues [3–5]. The tight binding of pyrazole in the ternary complex can be utilized to render NAD⁺ dissociation insignificant in active-site titrations of the enzyme [2] and in kinetic studies of coenzyme binding [6]. The high affinity of the enzyme · NAD⁺ complex for pyrazole makes it possible also to examine the kinetics of enzymic aldehyde reduction under single-turnover conditions by the 'catalytic suicide' method of McFarland and Bernhard [7].

Despite the extensive use of pyrazole for such methodological purposes in experiments carried out at different pH, detailed kinetic information about pyrazole binding to the enzyme · NAD⁺ complex is available only for the reaction at pH 7 [3]. The present investigation was undertaken to characterize the kinetics of the binding process over the wide pH range (4–10) usually considered in mechanistic studies of the enzyme. As will be illustrated in the following paper, such information is of primary interest for the design and

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD⁺ oxidoreductase (EC 1.1.1.1).

evaluation of transient-state kinetic experiments in which pyrazole is used as a reporter ligand. Secondly, examination of the kinetics of complex formation with pyrazole might provide inferences regarding the mechanism of substrate binding during liver alcohol dehydrogenase catalysis.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by McFarland and Bernhard [7]. When required, unbuffered enzyme solutions were prepared using 33 mM sodium sulphate for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [8] and are reported throughout as active-site concentrations.

The coenzymes NAD⁺ and NADH (Sigma Chemical Corp., grade III quality) were purified as described by Gurr et al. [9]. Pyrazole (Fluka AG) was recrystallized twice from water prior to use. Other reagents were of highest available quality and used without further purification.

All experiments reported in the present investigation were performed at 25°C in 0.1 M ionic strength phosphate (pH 6–10) or succinate (pH 4–6) buffer solutions, prepared using water twice distilled from a quartz apparatus.

Spectrometric Equipment and Data Processing

Reactions exhibiting half-times below 20 s were monitored photometrically by stop-flow techniques. The rapid-reaction

equipment described previously [10] was used, except that the Beckman DU monochromator was replaced by a Durrum D-130 monochromator. Reactions with half-times above 20 s were examined by standard spectrometric techniques using a Beckman Acta CIII spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer MPF-2A spectrofluorimeter.

Equilibrium constants for pyrazole binding to free enzyme and to the enzyme · NADH complex were determined fluorimetrically as described by Sigman et al. [11]. Rates and amplitudes of the exponential transients observed in the kinetic binding studies were estimated by non-linear regression analysis as detailed previously [10].

Pyrazole Association Rates

Liver alcohol dehydrogenase (about 10 μM) was preincubated with 4 mM NAD^+ in phosphate buffer of appropriate pH between 6 and 10. The equilibrium solution thus obtained (which can be assumed to contain at least 96% of the enzyme as the binary enzyme · NAD^+ complex [12]) was mixed in the stop-flow apparatus with an equal volume of the same buffer solution containing varied concentrations of pyrazole (0.04–10 mM). Apparent first-order rate constants for the exponential transient governing formation of the ternary enzyme · NAD^+ · pyrazole complex were determined from the absorbance changes observed at 300 nm [3].

Pyrazole Dissociation Rates

Liver alcohol dehydrogenase (about 10 μM) was preincubated with 4 mM NAD^+ and 60 μM pyrazole in phosphate buffer of appropriate pH between 6 and 8. Displacement of pyrazole from the ternary complex formed was then effected by the addition of a large excess of trifluoroethanol (50 mM after mixing) in the same buffer solution. Apparent first-order rate constants for the displacement reaction were determined from the absorbance changes observed at 300 nm, measurements being made by stop-flow techniques (pH 6–7) or conventional spectrophotometric techniques (pH 7–8).

The transient-state kinetics of the reverse reaction (displacement of trifluoroethanol from the enzyme · NAD^+ · alcohol complex by the addition of pyrazole) were examined over the pH range 4–6 by the pH-jump method described previously [5].

RESULTS

Pyrazole Association Rates

Eqn (1) shows the reaction scheme for pyrazole (P) binding to the binary complex (EO) formed between liver alcohol dehydrogenase and NAD^+ .



When pyrazole is present in large excess to enzyme, the kinetics of the binding process in Eqn (1) are governed by a single exponential transient. The corresponding apparent first-order rate constant (r) is given [13] by

$$r = k_{-1} + k_1[\text{P}]. \quad (2)$$

Estimates of r for the binding of varied concentrations of pyrazole to the enzyme · NAD^+ complex were determined

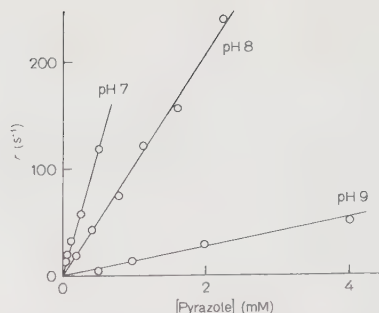


Fig. 1. Dependence on ligand concentration of the rate of pyrazole binding to the binary complex formed between liver alcohol dehydrogenase and NAD^+ . Apparent first-order rate constants (r) determined at 25°C in 0.1 M ionic strength phosphate buffer from the absorbance changes observed at 300 nm on reacting the enzyme (about 5 μM) with varied concentrations of pyrazole in the presence of 2 mM NAD^+ .

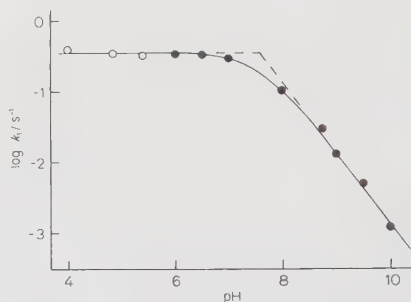


Fig. 2. Effect of pH on the on-velocity constant for pyrazole binding to the binary complex formed between liver alcohol dehydrogenase and NAD^+ . Estimates of the on-velocity constant (k_1) calculated from data such as, and including, those in Fig. 1 (●) and Fig. 5 (○). The curve drawn was calculated from Eqn (3) with $k_1^* = 350 \text{ s}^{-1} \text{ mM}^{-1}$ and $\text{p}K_7 = 7.6$.

by stop-flow techniques [3] at different pH between 6 and 10. As indicated by the typical results in Fig. 1, r was found to be linearly dependent on the pyrazole concentration at each fixed pH. Evaluation of the binding data in terms of Eqn (2) showed that the magnitude of k_{-1} was too small (less than 5 s^{-1}) to be reliably determined from these experiments. The estimates of k_1 obtained at different pH are given in Fig. 2 and conform to the relationship

$$k_1 = \frac{k_1^*}{1 + \frac{K_7}{[\text{H}^+]}} \quad (3)$$

with $k_1^* = 0.35 (\pm 0.05) \text{ s}^{-1} \mu\text{M}^{-1}$ and $\text{p}K_7 = 7.6 (\pm 0.2)$. It may be concluded from this result that pyrazole association to the enzyme · NAD^+ complex requires the protonated form of an ionizing group exhibiting a $\text{p}K_a$ of 7.6 in the binary complex, agreeing with the $\text{p}K_a$ value reported for the water molecule bound at the catalytic zinc ion of the enzyme subunit [1, 14, 15].

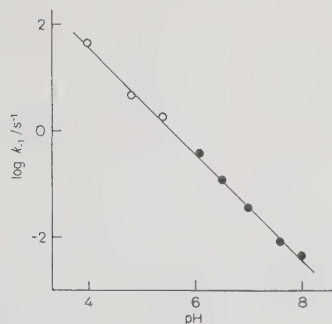


Fig. 3. Effect of pH on the off-velocity constant for pyrazole binding to the binary complex formed between liver alcohol dehydrogenase and NAD^+ . Estimates of the off-velocity constant (k_{-1}) calculated from data such as, and including, those in Fig. 5 (O). Values of k_{-1} for the pH range 6–8 (●) refer to the apparent first-order rate constant for the absorbance changes observed at 300 nm on mixing the enzyme (10 μM), preincubated with 4 mM NAD^+ and 60 μM pyrazole, with 100 mM trifluoroethanol at 25°C in 0.1 M ionic strength phosphate buffer. The line drawn has a slope of -1

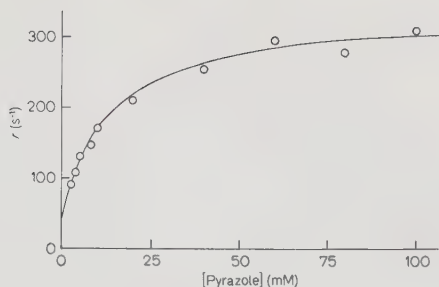
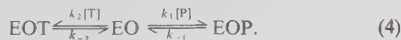


Fig. 4. Displacement of trifluoroethanol by pyrazole from the ternary complex formed with liver alcohol dehydrogenase and NAD^+ . Apparent first-order rate constants (r) calculated from the absorbance changes recorded at 300 nm on mixing enzyme (12 μM), preincubated at pH 6 with NAD^+ (4 mM) and trifluoroethanol (160 mM) in 33 mM sodium sulphate, with a 0.1 M ionic strength succinate buffer solution containing varied concentrations of pyrazole. The pH of the reaction solution after mixing was 4.0. The curve drawn represents a least-squares fit of Eqn (5) with the assumption that $k_2 = 60 \text{ s}^{-1} \text{ mM}^{-1}$ and $k_{-2} = 330 \text{ s}^{-1}$

Pyrazole Dissociation Rates

Eqn (4) shows the reaction scheme for a competitive [1,5] binding of pyrazole (P) and trifluoroethanol (T) to the enzyme $\cdot \text{NAD}^+$ complex (EO)



When ligand concentrations greatly exceed the total concentration of enzyme, the kinetics of the reaction system in Eqn (4) will be governed by two exponential transients [12]. Only the slower one of these transients would be expected to be detectable by stop-flow techniques, the corresponding apparent first-order rate constant (r) being given [12] by

$$r = \frac{k_{-2}k_1[\text{P}] + k_{-1}k_2[\text{T}] + k_{-1}k_{-2}}{k_1[\text{P}] + k_{-1} + k_2[\text{T}] + k_{-2}} \quad (5)$$

Determinations of the rate constant k_{-1} for pyrazole dissociation from the enzyme $\cdot \text{NAD}^+$ $\cdot$ pyrazole complex were performed by two different methods based on Eqn (5). In one series of experiments pyrazole was preincubated with the enzyme $\cdot \text{NAD}^+$ complex and displaced from the ternary complex formed by the addition of a large excess of trifluoroethanol. According to the binding data reported previously [5] and in the preceding section, ligand concentrations in these experiments were such ($[\text{P}] = 30 \mu\text{M}$ and $[\text{T}] = 50 \text{ mM}$) that Eqn (5) should reduce effectively to $r = k_{-1}$ [12], i.e. the observed rate of the displacement process can be assumed to provide a direct measure of the pyrazole dissociation rate. The estimates of k_{-1} thus obtained at different pH between 6 and 8 are given in Fig. 3 and show that the magnitude of k_{-1} is approximately proportional to the hydrogen ion concentration over the examined pH range. Above pH 8, the pyrazole dissociation rate became too low to be reliably determined.

In a second series of experiments trifluoroethanol was preincubated with the enzyme $\cdot \text{NAD}^+$ complex at pH 6. Displacement of the alcohol from the ternary complex formed was then effected at lower pH by the addition of varied con-

centrations of pyrazole. Fig. 4 shows the variation with pyrazole concentration of the apparent first-order rate constant r for the displacement reaction at pH 4. As has been previously reported [5], r increases non-linearly with increasing pyrazole concentration towards a maximum value (about 330 s^{-1}) representing the magnitude at pH 4 of the trifluoroethanol dissociation rate constant k_{-2} . The present extension of the measurements to much lower pyrazole concentrations makes it possible to arrive also at an extrapolated value (about 50 s^{-1}) of r at zero pyrazole concentration; according to Eqn (5), the latter value of r should provide an approximate measure of the pyrazole dissociation rate constant k_{-1} . More precise estimates of k_{-1} were determined by non-linear regression analysis. A fit of Eqn (5) to the data in Fig. 4 with the assumptions [5] that $k_2 = 60 \text{ s}^{-1} \text{ mM}^{-1}$ and $k_{-2} = 330 \text{ s}^{-1}$ gave $k_{-1} = 43 (\pm 6) \text{ s}^{-1}$ and $k_1 = 0.40 (\pm 0.07) \text{ s}^{-1} \mu\text{M}^{-1}$.

Estimates of the pyrazole dissociation rate constant thus obtained over the pH range 4–6 are included in Fig. 3. They show that the proportionality between k_{-1} and hydrogen ion concentration persists down to pH 4. The corresponding estimates of k_1 were found to be independent of pH and to differ insignificantly from the limiting value $k_1^* = 0.35 \text{ s}^{-1} \mu\text{M}^{-1}$ calculated from the data in Fig. 2. It can be inferred from these results that the relationships for pyrazole binding established by the measurements above pH 6 are valid also over the pH range 4–6.

The present estimates of k_{-1} (0.040 s^{-1}) and k_1 ($0.28 \text{ s}^{-1} \mu\text{M}^{-1}$) at pH 7 correspond to an equilibrium constant of $0.14 \mu\text{M}$ for pyrazole dissociation from the ternary complex. These binding data agree with those reported by Shore et al. [3] for the reaction at pH 7 ($k_1 = 0.284 \text{ s}^{-1} \mu\text{M}^{-1}$ and $k_{-1}/k_1 = 0.142 \mu\text{M}$).

Pyrazole Binding in the Absence of NAD^+

Equilibrium constants ($K_{\text{E,P}}$) for pyrazole dissociation from the binary enzyme $\cdot$ pyrazole complex were determined fluorimetrically using auramine O as a reporter ligand [11].

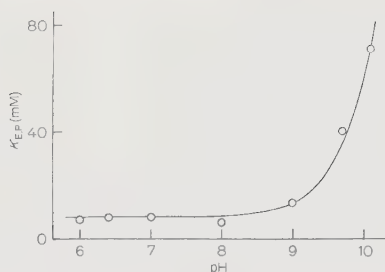


Fig. 5. Effect of pH on pyrazole binding to liver alcohol dehydrogenase in the absence of coenzymes. Equilibrium constants ($K_{E,P}$) for pyrazole dissociation from the binary complex formed with free enzyme determined fluorimetrically at 25 °C in 0.1 M ionic strength phosphate buffer using auramine O as a reporter ligand. The curve drawn was calculated from Eqn (6) with $K_{E,P}^* = 8$ mM and $pK_9 = 9.2$.

The estimates obtained at different pH between 6 and 10 are given in Fig. 5 and conform to the relationship

$$K_{E,P} = K_{E,P}^* \left(1 + \frac{K_9}{[H^+]} \right) \quad (6)$$

with $K_{E,P}^* = 8$ mM and $pK_9 = 9.2$. It may be concluded that pyrazole binding to free enzyme is dependent on a proton dissociation equilibrium with a pK_a value agreeing with that attributed to ionization of zinc-bound water in free enzyme [1, 15, 16].

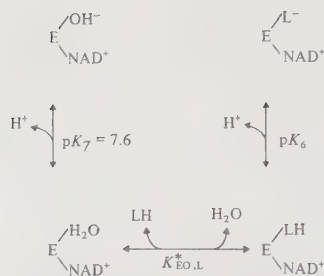
The equilibrium constant ($K_{E,P}$) for pyrazole dissociation from the enzyme $\cdot$ NADH $\cdot$ pyrazole complex was similarly determined at different pH between 6 and 10, which gave $K_{E,P} = 4 (\pm 1)$ mM independently of pH. No attempt was made to obtain estimates of $K_{E,P}$ over the pH range (10–12) where ionization of zinc-bound water in the enzyme $\cdot$ NADH complex becomes kinetically significant [17].

Displacement of pyrazole from the enzyme $\cdot$ NADH $\cdot$ pyrazole complex by the addition of varied concentrations of *N,N*-dimethylaminocinnamaldehyde showed no rate limitation detectable by stop-flow techniques. This observation indicates that the rate constant for pyrazole dissociation from the latter ternary complex exceeds 500 s^{-1} .

DISCUSSION

Mechanism of Pyrazole Binding

The catalytic zinc ion in the liver alcohol dehydrogenase subunit is bound by three protein ligands and coordinates a water molecule as a fourth inner-sphere ligand [1]. Ionization of this water molecule, which exhibits a pK_a value of 9.2 in free enzyme, 7.6 in the enzyme $\cdot$ NAD $^+$ complex, and 11.2 in the enzyme $\cdot$ NADH complex [1, 14–17], has been found to prevent complex formation between enzyme and external ligands which combine to the catalytic zinc ion [5, 14–17]. This is what might be expected if the external ligand is bound by a substitution mechanism with displacement of zinc-bound water [16]. The present investigation provides evidence that pyrazole binding to free enzyme (Fig. 5), as well as pyrazole association to the enzyme $\cdot$ NAD $^+$ complex (Fig. 2), is dependent on the ionization state of zinc-bound water with preferential binding to the unionized form. The observation that the affinity of pyrazole for the enzyme $\cdot$ NADH complex



Scheme 1. Generalized mechanism for the binding of an ionizing ligand to the binary complex formed between liver alcohol dehydrogenase and NAD $^+$. The ionizing ligand (LH) is assumed to combine to the catalytic zinc ion of the enzyme subunit (E) with displacement of zinc-bound water

remains unaffected by pH between pH 6 and 10 can be interpreted in the same way. These results lend strong support to the idea that pyrazole combines to the catalytic zinc ion on complex formation with the enzyme [1, 2], zinc-bound water most likely being displaced in the binding process.

The data in Fig. 3 establish that the rate constant for pyrazole dissociation from the enzyme $\cdot$ NAD $^+$ $\cdot$ pyrazole complex is approximately proportional to the hydrogen ion concentration between pH 4 and 8. It can be inferred from this observation that the ternary complex participates in an ionization step with a pK_a value below 4, pyrazole desorption occurring exclusively from the protonated form of the complex. This ionization step appears to be characteristic for the complex formed with pyrazole as the ligand. There is no similar effect of pH on the corresponding dissociation reaction with ligands such as decanoate [5] and 2,2'-bipyridine [15], which lack ionizing functional groups. Alcohol dissociation from the corresponding ternary complex, however, has been found to be controlled by ligand-dependent ionization steps with pK_a values within the range 4–7 [5, 14]. Kvassman and Pettersson [14] interpreted the latter effects of pH in terms of an alcohol/alcoholate ion equilibration of the enzyme-bound ligand, as indicated by the generalized binding mechanism in Scheme 1. They pointed out that alcoholate ion formation must be drastically facilitated by coordination of the ligand to the catalytic zinc ion and by electrostatic interaction with the positive charge on the nicotinamide ring of the coenzyme. Similar interactions have been proposed to account for the pK_a of zinc-bound water in the enzyme $\cdot$ NAD $^+$ complex [1, 12] and can be anticipated to be at hand and facilitate deprotonation of any ionizing ligand when bound to the catalytic zinc ion in the enzyme $\cdot$ NAD $^+$ complex. For these reasons, and since the kinetics of pyrazole binding to the latter complex are consistent with Scheme 1 (with $pK_6 < 4$), it seems reasonable to attribute the observed effect of pH on the pyrazole dissociation rate to ionization of the ligand at the ternary-complex level. This would explain why pyrazole desorption requires protonation of the ternary complex. The coulombic force field provided by the catalytic zinc ion and enzyme-bound NAD $^+$ will render the rate of dissociation of the negatively charged ligand negligibly small compared to the rate of desorption of the neutral pyrazole molecule.

According to the data in Fig. 5, pyrazole binding to free enzyme shows no pH dependence attributable to ionization of the ligand at the binary-complex level. This means that the

ionizing group with a pK_a value below 4 in the ternary complex must exhibit a pK_a value above 10 in the binary enzyme · pyrazole complex. The present results, therefore, provide additional evidence that the pK_a of an ionizing ligand may be drastically decreased on binding to the enzyme · NAD⁺ complex and indicate that the presence of NAD⁺ is a most important factor contributing to this pK_a perturbation.

The binding mechanism in Scheme 1 is consistent with the spectrometric and titrimetric data reported by Theorell et al. [2], who found that ternary-complex formation between enzyme, NAD⁺, and pyrazole results in a release of close to one proton per enzyme subunit at pH 7. Theorell et al. concluded from their results that N-2 of pyrazole combines to the active-site zinc ion, while N-1 of pyrazole was proposed to be covalently bound with liberation of a proton to C-4 of the nicotinamide ring of NAD⁺. We agree that the protons released on pyrazole binding to the enzyme · NAD⁺ complex most likely derive from the imino group of the ligand, but do not consider it necessary to assume that pyrazole and NAD⁺ are covalently linked in the ternary complex formed. Non-covalent interactions analogous to those proposed to account for the pK_a perturbations of zinc-bound water and alcohol ligands [14] might be sufficient to explain the increased acidity of the pyrazole imino group in the ternary complex.

Stability of the Enzyme · NAD⁺ · Pyrazole Complex

The data in Fig. 5 indicate that the affinity of free enzyme for pyrazole is of the same order of magnitude as the affinity for other inhibitory nitrogenous bases [1]. Pyrazole binding to the enzyme · NAD⁺ complex, however, appears to be exceptionally tight, the corresponding apparent equilibrium dissociation constant (k_{-1}/k_1) approaching a value of 0.03 μ M at high pH (Fig. 2 and 3). According to Scheme 1, the pH dependence of k_{-1}/k_1 can be expressed as

$$\frac{k_{-1}}{k_1} = K_{\text{EO,P}}^* \cdot \frac{1 + \frac{K_7}{[\text{H}^+]}}{1 + \frac{K_6}{[\text{H}^+]}} \quad (7)$$

where $K_{\text{EO,P}}^*$ denotes the true equilibrium constant for dissociation of the neutral pyrazole molecule from the ternary complex. Eqn (7) shows that the tight binding of pyrazole at high pH could be due either to a high value of K_6 (stabilization through ionization of the bound ligand) or to a low value of $K_{\text{EO,P}}^*$ (exceptionally firm binding of the neutral ligand) or both.

The present kinetic studies have not been carried out at sufficiently low pH to provide any direct estimates of the constants K_6 and $K_{\text{EO,P}}^*$. Assuming that $pK_7 = 7.6$ (Fig. 2), however, it follows from Eqn (7) and the present estimates of k_{-1}/k_1 (0.14 μ M at pH 7) that the two constants are inter-related approximately through

$$K_{\text{EO,P}}^* = 1.1 K_6. \quad (8)$$

Since the results in Fig. 3 establish that $pK_6 < 4$, it may be concluded from Eqn (8) that $K_{\text{EO,P}}^* > 0.1$ mM. This means that binding of the neutral pyrazole molecule to the enzyme · NAD⁺ complex is stabilized by less than 1.9 pK (corresponding to 11 kJ/mol) compared to pyrazole binding to free enzyme ($K_{\text{E,P}}^* = 8$ mM) and to the enzyme · NADH complex ($K_{\text{ER,P}} = 4$ mM).

Two main inferences can be drawn from this observation. Firstly, it seems obvious that the exceptional stability of the enzyme · NAD⁺ · pyrazole complex at high pH ($k_{-1}/k_1 \approx 0.03$ μ M) is attributable mainly or entirely to the low magnitude of pK_6 , i.e. to ionization of the ligand resulting from the drastic pK_a perturbation brought about by complex formation. The low pK_a value for this ionization process accounts for the steady decrease in stability of the ternary complex with increasing hydrogen ion concentration over the examined pH range. The equilibrium dissociation constant k_{-1}/k_1 is of less remarkable magnitude (about 100 μ M) at pH 4, and might well approach a limiting value exceeding 1 mM at extremely low pH. This would require that the limiting value at low pH of the corresponding off-velocity constant (Fig. 3) exceeds 350 s⁻¹. Strong evidence that such is actually the case is provided by the observation that pyrazole dissociation from the ternary complex formed with reduced coenzyme occurs at an immeasurably high rate (> 500 s⁻¹).

Secondly, it may be noted that an energetic binding contribution of maximally 11 kJ/mol would be considerably smaller than that provided by an ordinarily weak hydrogen bond (20–30 kJ/mol). The possibility that covalent interactions with NAD⁺ contribute to the binding of pyrazole in the neutral state, therefore, is rendered highly unlikely by the present results. It then seems reasonable (and is justified by data reported for substrate binding to the enzyme [14]) to assume that interactions of similar nature and strength contribute to the binding of the neutral pyrazole molecule in the ternary complexes formed with NAD⁺ and NADH. In other words, the value (4 mM) observed for $K_{\text{ER,P}}$ below pH 10 can be taken to provide an approximate measure of the actual magnitude of $K_{\text{EO,P}}^*$. According to Eqn (8), this estimate of $K_{\text{EO,P}}^*$ corresponds to $pK_6 = 2.4$ and hence to a NAD⁺-induced perturbation of the pK_a of enzyme-bound pyrazole by more than 7 pK. A pK_a perturbation of that order of magnitude would not be unreasonably large to derive mainly from electrostatic effects of the positively charged nicotinamide ring of enzyme-bound NAD⁺.

Irrespective of the detailed nature of the interactions contributing to stabilization of the ionized state of the ligand in the ternary complex, the present investigation seems to establish that the mechanism of pyrazole binding to the enzyme · NAD⁺ complex is closely analogous to that of the binding of alcohol ligands. This provides additional justification for the methodological use of pyrazole as a chromophoric substrate analogue, at least in applications where the low dissociation rate of pyrazole at alkaline pH poses no problems. In particular, the kinetic data now reported will make it possible to use pyrazole as a well-characterized reporter ligand in transient-state kinetic studies of the binding of non-chromophoric alcohols. The value of the present results in the latter respect will be illustrated in the following paper.

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Synergism between Coenzyme and Aldehyde Binding to Liver Alcohol Dehydrogenase

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1. Aldehyde binding to liver alcohol dehydrogenase in the absence and presence of coenzymes has been characterized by spectrometric equilibrium methods, using auramine O and bipyridine as reporter ligands.

2. Free enzyme shows a significant affinity for aldehydes, and equilibrium constants for dissociation of the binary complexes formed with typical aldehyde substrates are reported. Binary-complex formation does not lead to any detectable inner-sphere coordination of aldehydes to the catalytic zinc ion of the enzyme subunit.

3. Complex formation with NAD^+ or NADH increases the affinity of the enzyme for aromatic aldehydes by a factor of 1.8–3.5 and 6–17, respectively. Benzaldehyde and dimethylaminocinnamaldehyde binding to the enzyme $\cdot \text{NAD}^+$ complex is not detectably associated with inner-sphere coordination of the aldehyde to zinc.

4. It is concluded that binding of NADH is required to induce catalytically adequate bonding interactions between enzyme and aromatic aldehydes. The effect of reduced coenzyme in this respect is attributed to hydrophobic interactions leading to dehydration of the active-site region, which allows aldehyde substrates to compete successfully with water for inner-sphere coordination to the catalytic zinc ion. Oxidized coenzyme is proposed to have a similar promoting effect on metal coordination of aldehydes which function as substrates for the dismutase activity of the enzyme.

Liver alcohol dehydrogenase [1] catalyzes alcohol/aldehyde interconversion by a ternary-complex mechanism with NAD^+ /NADH as coenzymes. The enzymic reduction of aldehydes invariably has been found to be kinetically ordered with coenzyme binding preceding substrate binding [1–4]. This could indicate that the enzyme shows an insignificantly low affinity for aldehyde substrates in the absence of NADH [5]. Apart from a preliminary (unconfirmed) report that acetaldehyde and butyraldehyde are bound in competition with the zinc-chelating reagent 2,2'-bipyridine [6], there is no direct evidence that aldehydes interact detectably with free enzyme. On the other hand, strong synergistic effects of coenzyme on the binding of substrate analogues have been documented in several instances [1, 5], so the affinity of liver alcohol dehydrogenase for aldehydes might well increase drastically on complex formation with NADH.

Evidence consistent with the latter idea has been reported by Dunn and Hutchison [4]. They found that enzyme, NADH, and *trans*-*N,N*-dimethylaminocinnamaldehyde (DACA) form a catalytically competent ternary complex with spectral properties indicative of aldehyde binding to the catalytic zinc ion of the enzyme subunit. No similar chromophoric complex was formed in the absence of coenzyme or when NADH was replaced by NAD^+ . As pointed out by Dunn [5], this observation might indicate that NADH is specifically required to obtain a detectably tight binding of DACA, which would imply that reduced coenzyme acts as a strong positively cooperative effector of aldehyde binding. Alternatively, NADH may be of specific importance mainly for the induction of bonding interactions which render complex formation with DACA detectably chromophoric. Lack of knowledge about the actual

affinity of DACA for free enzyme and for the enzyme $\cdot \text{NAD}^+$ complex precludes unambiguous interpretation of the interesting results of Dunn and Hutchison in these mechanistically critical respects.

Similar considerations apply for complex formation between enzyme and aldehydes in general. No data are available which establish the existence, or illustrate the actual magnitude and oxidation-state dependence, of synergistic effects of coenzymes on aldehyde binding to the enzyme or aldehyde bonding to the catalytic zinc ion. This seems remarkable, considering that such effects relate directly to the energetics of productive ternary-complex formation, and hence must reflect interactions of catalytic significance.

The present investigation was undertaken to obtain such data. We have now examined the synergism between coenzyme and aldehyde binding to liver alcohol dehydrogenase with particular reference to (heterotropic) cooperativity effects and zinc coordination of the aldehyde ligand. Results now reported clarify in which respects, and to what extent, enzyme-bound NADH may be considered to promote the productive binding of aldehyde substrates. The structural and energetic factors leading to metal coordination of aldehydes in productive ternary complexes are discussed in detail.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [8], except that 50 mM phosphate buffer was used for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of

Abbreviation. DACA, *trans*-*N,N*-dimethylaminocinnamaldehyde.

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

isobutyramide [9] and are reported throughout as active-site concentrations.

The coenzymes NAD^+ and NADH (Sigma Chemical Corp., grade III quality) were purified as described by Gurr et al. [10]. Aldehydes were obtained from BDH Chemicals (Poole) and either sublimed or vacuum distilled prior to use. Other reagents were of highest available quality and used without further purification. Auramine O, 2,2'-bipyridine, and $\text{K}_2\text{Pt}(\text{CN})_4$ were purchased from Fluka AG (Buchs), AMP and ADP-ribose from Sigma Chemical Corp. (St Louis, MO).

Methods

Equilibrium constants for aldehyde binding to liver alcohol dehydrogenase were determined fluorimetrically as described by Sigman and Glazer [11] and further detailed by Andersson et al. [12]. Fluorescence changes associated with binding of the reporter ligand auramine O to the enzyme were monitored at 530 nm (excitation at 430 nm) using a Perkin-Elmer MPF-2A spectrofluorimeter. Solutions containing about 0.3 μM auramine O and varied concentrations of aldehyde were titrated with enzyme in the absence or presence of ligands which combine to the coenzyme-binding site of the enzyme subunit. Concentrations of the latter ligands were high enough [0.2 mM NADH, 2 mM NAD^+ , 2 mM ADP-ribose, 5 mM AMP, 6 mM $\text{Pt}(\text{CN})_4^{2-}$] to be more than 97% saturating according to previously reported binding data [13, 14].

Effects of aldehydes on complex formation between enzyme and 2,2'-bipyridine were examined at pH 7 and 8.8 as described by Sigman [6]. A Shimadzu UV-240 spectrophotometer was used in these experiments. Difference absorption spectra for complex formation between enzyme and dimethylaminocinnamaldehyde were recorded with a Beckman Acta CIII spectrophotometer.

All measurements were made at 25°C in 0.1 M ionic strength phosphate (pH 6–8.8) or carbonate (pH 9–10) buffer. Dissociation constant estimates reported in Tables 1–3 represent the mean of two, three or four independent determinations, standard deviations of the estimates being typically about 20% of the values given.

RESULTS

Aldehyde Dissociation Constants at pH 8.8

The binding to liver alcohol dehydrogenase of DACA and more typical aromatic or aliphatic substrates was examined by spectrofluorimetric equilibrium methods, using auramine O as a reporter ligand [11]. All aldehydes tested were found to displace auramine O competitively from the enzyme, indicating that binary enzyme · aldehyde complexes do form at experimentally accessible substrate concentrations. Equilibrium constants ($K_{\text{E,Ald}}$) for dissociation of the binary complexes were determined at pH 8.8 as described by Andersson et al. [12] and the results are listed in Table 1.

Equilibrium constants $K_{\text{EL,Ald}}$ for aldehyde dissociation from ternary enzymic complexes were similarly determined at pH 8.8 by displacement of auramine O in the presence of saturating concentrations of ligands (L) which combine to the coenzyme-binding site of the enzyme subunit (Tables 1 and 2). DACA binding to the enzyme was found to be tightened about six-fold in the presence of NADH. Substrate dissociation constants for catalytically more rapidly reacting enzyme · NADH · aldehyde complexes could not be determined by the

Table 1. Aldehyde binding to liver alcohol dehydrogenase

Equilibrium constants for aldehyde dissociation from the enzyme in the absence ($K_{\text{E,Ald}}$) and presence of saturating concentrations of NAD^+ ($K_{\text{ENAD}^+, \text{Ald}}$) or NADH ($K_{\text{ENADH, Ald}}$), determined at 25°C in 0.1 M ionic strength phosphate buffer (pH 8.8). Values within brackets refer to kinetic estimates of $K_{\text{ENADH, Ald}}$ [15–17] for catalytically productive complexes

Aldehyde	Dissociation constant		
	$K_{\text{E,Ald}}$	$K_{\text{ENAD}^+, \text{Ald}}$	$K_{\text{ENADH, Ald}}$
	μM		
DACA	35	14	6
Naphtaldehyde	450	130	(30)
p-Methoxy-benzaldehyde	6000	3000	(350)
Benzaldehyde	6000	3400	(400)
Butyraldehyde	10000	—	—
Acetaldehyde	110000	—	—

Table 2. Cooperativity between aldehyde binding and complex formation at the coenzyme-binding site

Equilibrium constants ($K_{\text{EL,Ald}}$) for benzaldehyde and DACA dissociation from different ternary complexes. Conditions as in Table 1

Ligand (L)	Benzaldehyde		DACA	
	$K_{\text{EL,Ald}}$	$K_{\text{E,Ald}}$	$K_{\text{EL,Ald}}$	$K_{\text{E,Ald}}$
	μM		μM	
None	6000	1.0	35	1.0
$\text{Pt}(\text{CN})_4^{2-}$	5400	1.1	30	1.2
AMP	4800	1.3	23	1.5
ADP-ribose	3700	1.6	25	1.4
NAD^+	3400	1.8	14	2.5
NADH	(400)	15	7	5.0

present equilibrium methods, but have been previously estimated for some aromatic aldehydes by transient-state kinetic methods [15–17]. It follows from the latter estimates and results now obtained (Table 1) that complex formation with NADH increases the affinity of the enzyme for these aldehydes by a factor of 15–17.

Complex formation with NAD^+ tightens the binding of aromatic aldehydes examined by a factor of 1.8–3.5 (Table 1). The effect of NAD^+ on the binding of aliphatic aldehydes could not be reliably determined due to the dismutase activity of the enzyme towards such substrates [18].

Table 2 shows that a less marked synergistic stabilization of aldehyde binding occurs on ternary-complex formation with various coenzyme fragments or with the coenzyme-competitive anion $\text{Pt}(\text{CN})_4^{2-}$ [19]. This indicates that the pronounced cooperativity in the binding of aldehydes and NADH derives from interactions induced mainly by binding of the nicotinamide portion of reduced coenzyme.

Effect of pH on Aldehyde Binding

Inner-sphere coordination of substrates or inhibitory reagents to the catalytic zinc ion of the enzyme subunit invariably has been found to require the protonated state of an ionizing enzymic group which has been identified as zinc-bound water

Table 3. Effect of pH on aldehyde binding to free enzyme and to the enzyme $\cdot$ NAD $^{+}$ complex

Conditions as in Table 1, except that measurements were made at different pH in 0.1 M ionic strength phosphate (pH 6–8.8) or carbonate (pH 9–10) buffer

pH	Benzaldehyde		DACA	
	$K_{E,Ald}$	$K_{ENAD^{+},Ald}$	$K_{E,Ald}$	$K_{ENAD^{+},Ald}$
	mM		μ M	
6	6.4	3.0	30	13
7	7.2	2.6	35	13
8	5.2	3.0	32	16
8.8	6.0	3.4	35	14
9	6.3	3.3	32	17
10	5.2	2.8	38	12

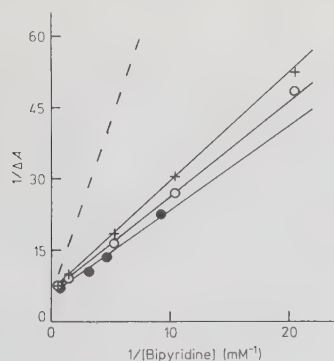


Fig. 1. Effect of aldehydes on bipyridine binding to liver alcohol dehydrogenase. Double-reciprocal plot of absorbance changes (ΔA) recorded on titration of the enzyme (15 μ M) with bipyridine in the absence (+) and presence of 12 mM benzaldehyde (○) or 20 mM butyraldehyde (●). The dashed line shows the titration curve expected for bipyridine binding in competition with the aldehydes

[1, 12]. This group exhibits pK_a values of 9.2 and 7.6 in free enzyme and the enzyme $\cdot$ NAD $^{+}$ complex, respectively [12, 14]. Results presented in Table 3 show that DACA and benzaldehyde binding to the latter two enzymic species remains unaffected by pH changes over the pH range 6–10 and hence must be essentially independent of the ionization state of zinc-bound water. This observation provides strong evidence that there is no significant inner-sphere coordination to zinc of the substrate in the enzyme $\cdot$ aldehyde and enzyme $\cdot$ NAD $^{+}$ $\cdot$ aldehyde complexes formed with DACA and benzaldehyde.

Effect of Aldehydes on Bipyridine Binding

The inhibitor 2,2'-bipyridine, which forms a chromophoric chelate with catalytic zinc on complex formation with liver alcohol dehydrogenase [1], has been reported to be competitively displaced by acetaldehyde and butyraldehyde [6]. We have not been able to confirm that observation. Spectrophotometric titration data plotted in Fig. 1 show that the

Furthermore, no absorption changes are associated with the apparent affinity of the enzyme for bipyridine actually increases slightly (certainly does not decrease) in the presence of 20 mM butyraldehyde or 12 mM benzaldehyde, i.e. with aldehyde concentrations in two-fold excess to the respective $K_{E,Ald}$ values in Table 1. Analogous results were obtained with other aldehydes listed in Table 1. These observations definitely establish that aldehydes do not combine directly to the catalytic zinc ion on binary-complex formation with the enzyme, at least not in the specific interaction which leads to competitive displacement of auramine O.

Chromophoric Properties of DACA Complexes

Difference absorption spectra were recorded at pH 8.8 for enzyme solutions containing about 75% of the enzyme $\cdot$ DACA complex or 90% of the enzyme $\cdot$ NAD $^{+}$ $\cdot$ DACA complex according to binding data in Table 1. No absorption differences attributable to the presence of these complexes could be detected in the 300–700-nm region. This confirms the observations made by Dunn and Hutchison [7] and establishes that DACA binding is not necessarily associated with appearance of the characteristic 464-nm absorption band of the enzyme $\cdot$ NADH $\cdot$ DACA complex.

DISCUSSION

Effect of Coenzymes on Aldehyde Binding

The equilibrium constant estimate reported in Table 1 for aldehyde dissociation from the chromophoric ternary complex formed between liver alcohol dehydrogenase, NADH, and DACA agrees with that determined by spectrophotometric methods [7, 20]. This confirms previous reports that auramine O can be reliably used as a reporter ligand to monitor catalytically relevant interactions at the substrate-binding site of the enzyme subunit [11, 21]. The present results, therefore, provide clear evidence that aldehydes combine to the substrate-binding site whether or not the coenzyme-binding site is occupied.

The affinity of the enzyme for aromatic aldehydes now examined increases significantly on coenzyme binding, the stabilizing effect of NADH being considerably larger than that of NAD $^{+}$ (Tables 1 and 2). The affinity increase caused by NADH binding may be of physiological importance as a partial determinant of the K_m value for aldehyde substrates, but there is otherwise little reason to emphasize the mechanistic implications of this synergistic effect. In particular, the idea must be rejected that the synergism between coenzyme and substrate binding conveys a discernable kinetic order to their binding during catalysis [5]. If NADH binding stabilizes complex formation with aldehydes to a certain extent, then aldehyde binding must stabilize complex formation with NADH to exactly the same extent and the net effect on the order of catalytic NADH/aldehyde binding will be nil.

Effect of Coenzymes on Aldehyde Binding

The effect of coenzymes on bonding interactions between enzyme and aldehyde substrates appears to be of greater mechanistic interest. Data now reported establish that there is no detectable inner-sphere coordination of aldehydes to catalytic zinc in the binary enzyme $\cdot$ aldehyde complexes formed with substrates listed in Table 1, or in the enzyme $\cdot$ NAD $^{+}$ $\cdot$ aldehyde complexes formed with DACA and benzaldehyde.

binding of DACA to free enzyme or to the enzyme $\cdot$ NAD⁺ complex. These observations corroborate the conclusion of Dunn and Hutchison that the 464-nm absorption band of the enzyme $\cdot$ NADH $\cdot$ DACA complex is attributable to direct zinc coordination of the aldehyde [7, 22] and rule out alternative explanations for the appearance of this absorption band.

Dunn interpreted the absolute requirement for reduced coenzyme in formation of the chromophoric DACA complex as a manifestation of oxidation-state-dependent differences in affinity of the enzyme $\cdot$ coenzyme complexes for DACA [5]. The present results exclude that possibility and show that the requirement for NADH reflects effects on DACA bonding rather than DACA binding. The stabilizing effects of NADH and NAD⁺ on DACA binding are not drastically different (Table 2), but chromophoric inner-sphere coordination of the aldehyde to zinc occurs only in the ternary complex formed with reduced coenzyme.

There is strong reason to believe that aldehydes are generally ligated to the catalytic zinc ion in the productive ternary complexes formed during catalysis [1, 22–24]. The present investigation establishes that NADH binding is specifically required with some substrates for induction of this mechanistically crucial interaction.

Origin of Observed Synergistic Effects

Hydrophobic interactions (the net effect of a multitude of solvent/protein/ligand interactions) are known to provide a major driving force for complex formation at the substrate-binding site of the liver alcohol dehydrogenase subunit [1]. The present results show that aldehyde binding to this site is additionally stabilized by simultaneous occupation of the adjacent nicotinamide-binding part of the coenzyme-binding site. The synergistic stabilization obtained is comparatively weak, however. The cooperativity factors (6–17) observed for NADH and aldehyde binding correspond to a shift in free energy change of 4–8 kJ/mol, which is about one-fifth of the energetic contribution provided by formation of an ordinarily strong hydrogen bond. This indicates that the synergistic stabilization derives from energetically subtle changes in the binding situation, changes which most likely can be related mainly to hydrophobic interactions at the junction of the two binding sites. In simplified terms, complex formation at one site (and concomitant solvent displacement from this site) can be envisaged to render the adjacent site more hydrophobic and hence more tightly binding. This explains why no pronounced synergistic effects are associated with the binding of aldehydes and coenzyme analogues which lack the nicotinamide portion of the coenzyme (Table 2). Attribution of the observed synergism to hydrophobic interactions is consistent also with recent crystallographic results [22], which show that ternary-complex formation between enzyme, DACA, and tetrahydro-NADH leads to an extensive exclusion of water molecules from the active-site region.

The observed zinc coordination pattern for complex formation with aldehydes can be interpreted in similar terms. Water shows a higher intrinsic affinity for metal ions than does the aldehyde carbonyl group. Aldehyde ligands, therefore, would be expected to compete poorly with water for metal coordination in binary enzyme $\cdot$ aldehyde complexes, where the catalytic zinc ion is accessible for interaction with water molecules from solvent via the unoccupied coenzyme-binding site [1, 24]. This explains why aldehyde binding to free enzyme does not lead to any detectable inner-sphere zinc coordination of the ligand.

In complexes containing bound coenzyme the catalytic zinc ion is accessible from solution exclusively via the substrate-binding site, and only when this site is unoccupied [1, 24]. The intrinsically unfavourable substitution of zinc-bound water by the aldehyde carbonyl group may then be energetically compensated by transfer of the water molecule from the largely hydrophobic interior of the protein to solvent. Complex formation with NADH (and concomitant changes in protein conformation [1, 24]) appears to render the environment of the catalytic zinc ion sufficiently hydrophobic to ensure that zinc-bound water is actually displaced and expelled on aldehyde binding. Complex formation with NAD⁺ introduces a positive charge close to the catalytic zinc ion, which favours retention of water in the active-site region (e.g. to diminish repulsive charge interactions). This appears to shift the energetic balance and render inner-sphere coordination insignificant for the aromatic aldehydes now examined. Partial retention of water is a likely explanation for the lower stabilizing effect of NAD⁺ (compared to NADH) on the binding of aromatic aldehydes.

General Conclusions

The zinc coordination pattern observed for complex formation with benzaldehyde and DACA is likely to be typical for aromatic aldehydes, but should not be taken as evidence that reduced coenzyme is specifically required to induce metal coordination of aldehyde substrates in general. Aromatic aldehydes show a low tendency for carbonyl group hydration. Formaldehyde and homologous aliphatic aldehydes, however, are known to form significant amounts of hydrates in solution [25]. Ligation of a hydrated aldehyde to the catalytic zinc ion would retain the main energetic advantages of a zinc-bound water molecule, and might well occur also in the presence of oxidized coenzyme.

A hydrated aldehyde is a divalent alcohol and would actually be expected [25] to be bound and catalytically oxidized in the presence of NAD⁺ by the mechanism established for the enzymic oxidation of monovalent alcohols [22], i.e. through coordination to zinc followed (and stabilized) by alcoholate ion formation and subsequent hydride transfer to NAD⁺. The fact that the enzyme shows an aldehyde oxidase (or dismutase) activity towards many aliphatic aldehydes can thus be readily understood in view of the present results, which indicate that the metal coordination state of the aldehyde is an important factor controlling the substrate specificity and turnover rate of the enzyme with respect to the latter activity.

There is little reason to draw any categoric inferences about effects of coenzymes on the interaction of aldehydes with catalytic zinc in terms of absence or presence of inner-sphere coordination of the substrate. The coordination state of an enzyme-bound aldehyde appears to be determined by a delicate energetic balance, and could thus be differently affected by coenzyme binding depending on the detailed steric and electronic properties of the aldehyde (including its tendency for carbonyl group hydration). Furthermore, it would seem preferable in many contexts to consider the coordination state of bound aldehydes as a matter of quantity rather than quality. The enzyme may act catalytically upon an aldehyde even if only a minor molar fraction of the enzyme-bound substrate is adequately metal-coordinated, and there is probably always an equilibrium distribution of bound aldehydes between the inner and outer coordination spheres of the catalytic zinc ion. The observation that the enzyme shows a weak dismutase activity towards certain aromatic aldehydes [26] can be readily explained in such terms.

On the other hand, it seems reasonable to assume on evolutionary grounds that inner-sphere zinc coordination of aldehydes is generally predominant in productive ternary complexes formed with physiologically important substrates. The present results, further, seem to establish that inner-sphere coordination is generally insignificant in the binary enzyme-aldehyde complexes. So, independently of whether aldehyde reduction by NADH or aldehyde oxidation by NAD⁺ represents the more interesting activity of the enzyme from a physiological point of view, complex formation with coenzyme must be considered as a mechanistically important factor promoting aldehyde binding in a catalytically productive (zinc-ligated) mode. The role played by coenzyme in this respect can be related to synergistic dehydration of the active-site region, which allows the aldehyde to compete successfully with water for catalytic zinc ion. The necessity of ensuring extensive inner-sphere coordination to zinc of aldehyde substrates may be a primary reason why the enzyme is structurally designed [22, 24] to react bound substrate with bound coenzyme in a largely anhydrous protein environment.

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Catalytic significance of binary enzyme-aldehyde complexes in the liver alcohol dehydrogenase reaction

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1. The interaction of liver alcohol dehydrogenase with NADH and aldehyde substrates has been characterized with respect to ternary-complex formation by the apparently non-preferred pathway which involves intermediate formation of binary enzyme·aldehyde complexes. Rate constant estimates are reported for dimethylaminocinnamaldehyde (DACA) binding to free enzyme and for NADH binding to the enzyme·DACA complex.

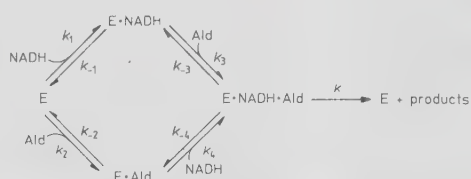
2. The rate of NADH (or NAD^+) association to liver alcohol dehydrogenase is not detectably affected by DACA binding to the enzyme, but the NADH dissociation rate decreases approximately by a factor of 6. The NADH-induced increase in affinity of the enzyme for DACA is similarly attributable to a decreased dissociation rate rather than an increased association rate of the aldehyde. DACA dissociates much more rapidly than coenzyme from the enzyme·NADH·aldehyde complex and shows a higher association rate constant than NADH in its interaction with free enzyme.

3. It is concluded from these results that the enzymic reduction of typical aldehyde substrates will conform to a rate equation which is experimentally indistinguishable from that of a compulsory-order mechanism with coenzyme binding preceding substrate binding, and that this rate equation will obtain irrespective of which pathway for ternary-complex formation is actually preferred. Rate equations provide no reliable information about the order of ligand binding in ternary-complex systems.

4. A flow analysis is presented which indicates that coenzyme and substrate are actually bound in random order to liver alcohol dehydrogenase during the enzymic reduction of aldehydes by NADH. The enzyme·aldehyde pathway for ternary-complex formation is fully kinetically competent, and reaction flow *via* this pathway may predominate when aldehyde concentrations exceed those required for half-saturation of free enzyme. Binary enzyme·aldehyde complexes are seemingly insignificant with respect to the rate behaviour of the enzyme, but may provide most significant and even predominant contributions to the catalytic reaction flow.

Liver alcohol dehydrogenase [1] catalyzes alcohol/aldehyde interconversion by a ternary-complex mechanism with NAD^+/NADH as coenzymes. The enzyme shows a significant affinity for aldehyde substrates whether or not NADH is present (and vice versa) [1,2], which indicates that the productive enzyme·NADH·aldehyde complexes can be assumed to be formed by a basically random-order binding mechanism (Scheme 1). The enzyme·aldehyde pathway for ternary-complex formation in Scheme 1 does not appear to contribute appreciably to the catalytic reaction flow, however. The enzymic reduction of aldehydes by NADH invariably has been found to conform to the rate equation of a compulsory-order mechanism with coenzyme binding preceding binding of the aldehyde substrate [3–6].

The reason why this binding order appears to be preferred remains obscure. Essentially no information is available about the effect of coenzyme on the kinetics of substrate binding, or about the effect of substrates on the kinetics of coenzyme binding. Consequently, we cannot tell if NADH binding facilitates the interaction of enzyme with aldehydes, or if aldehyde binding affects coenzyme association to the enzyme negatively, or if the enzyme·aldehyde pathway is rendered kinetically insignificant due to a more complex interplay of



Scheme 1. Simplified random-order mechanism for liver alcohol dehydrogenase catalysis of aldehyde (Ald) reduction by NADH

rate and equilibrium constants for reaction steps in Scheme 1. It would be of obvious mechanistic interest to obtain some detailed knowledge at these points. In particular, such knowledge might lead to a better understanding of the nature of the synergistic substrate-coenzyme interactions which are known to stabilize the enzyme·NADH·aldehyde complexes and to be essential for the catalytic competence of these complexes [2].

It is of interest also from a theoretical and methodological point of view to clarify why the enzyme·aldehyde pathway for ternary-complex formation does not contribute detectably to the rate equation for enzymic aldehyde reduction. The interpretation of reaction kinetics in ternary-complex systems is a matter of considerable complexity; uncertainty still persists

Abbreviation. DACA, *trans*-N,N-dimethylaminocinnamaldehyde.

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

regarding fundamental mechanistic implications of the observed rate behaviour of liver alcohol dehydrogenase. The mere fact that enzymic aldehyde reduction conforms to a compulsory-order type of rate equation cannot be uncritically taken to indicate that the actual reaction flow is correspondingly ordered (not even preferentially). This interpretational ambiguity may not be generally recognized, but is a reality according to theoretical analyses of the rate and flow behaviour of random-order ternary-complex systems [7,8].

Understanding of the mechanism of action of liver alcohol dehydrogenase in respects considered above requires detailed kinetic information about the apparently insignificant (and hence previously unexamined) pathway for ternary-complex formation in Scheme 1, i.e. the one involving formation of, and coenzyme binding to, the binary enzyme-aldehyde complex. We have now carried out a series of transient-state kinetic measurements which provide such information for the reaction between enzyme, NADH, and *trans*-*N,N*-dimethylaminocinnamaldehyde (DACA). Arguments are presented which indicate that results obtained with DACA are typical in certain qualitative respects for aldehyde binding in general. Assuming that such is the case, the present investigation clarifies the kinetic origin of the synergism between aldehyde and NADH binding, explains the rate behaviour of the enzyme in its action upon aldehyde substrates, and provides an unexpected inference as to the catalytic significance of binary enzyme-aldehyde complexes.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [9], except that 50 mM phosphate buffer was used for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [10] and are reported throughout as active-site concentrations.

The coenzymes NAD⁺ and NADH (Sigma Chemical Corp., grade III quality) were purified as described by Gurr et al. [11]. *trans*-*N,N*-Dimethylaminocinnamaldehyde (BDH Chemicals, Poole) was purified by vacuum sublimation prior to use. Auramine O and 2,2'-bipyridine were purchased from Fluka AG (Buchs) and used without further purification.

All measurements were made at 25°C in 0.1 M ionic strength phosphate buffer, pH 9.0, prepared using twice-distilled water.

Rapid-reaction equipment and data evaluation

Transient-state kinetic measurements were made with a Durrum D-100 stopped-flow photometer system. Photomultiplier signals were visualized in a Tektronix 5103N storage oscilloscope and were registered and digitalized in a Datalab DL 905 transient recorder. Registered data were written out on a Hewlett-Packard 7005B XY-recorder, or were read into a GNAT System 10 computer for statistical evaluation. The dead-time of the stopped-flow instrument was estimated to 2.3 ms by the method described by Gutfreund [12].

Apparent first-order rate constants for recorded transients were determined by non-linear regression methods as detailed previously [13].

Fluorimetric measurements

The kinetics of dimethylaminocinnamaldehyde (DACA) binding to liver alcohol dehydrogenase were examined by displacement methods, using auramine O as reporter ligand [14]. A 40 μ M solution of one of the two competitively bound ligands was pre-equilibrated with about 4 μ M enzyme in one syringe, and was mixed with an excess of the other ligand (50–250 μ M DACA or 100–800 μ M auramine O) added from the second syringe. Reactions were performed in the absence of coenzyme, as well as in the presence of at least 99.95 % saturating concentrations of NADH (1–5 mM), premixed with enzyme. Concentrations given above are those before mixing in the stopped-flow apparatus. Concentrations stated elsewhere in this paper refer to those of the actual reaction solution after mixing.

The displacement of auramine O initiated by the addition of DACA (and vice versa) was monitored fluorimetrically. The excitation wavelength was 430 nm; a Corning CS 3-69 filter was used to avoid registration of light emitted at wavelengths below 470 nm. Photomultiplier signals for total fluorescence changes associated with changes in the concentration of bound auramine O (typically about 1 μ M) during reactions were strong enough to be adequately recorded without amplification. Due to the favourable signal-to-noise ratio, the minimum rate of transients occurring mainly during the dead-time of the instrument could be estimated with great precision.

The equilibrium constant for auramine O dissociation from the ternary enzyme-NADH-auramine O complex was determined as described by Sigman et al. [14].

Photometric measurements

The transient-state kinetics of NADH association to liver alcohol dehydrogenase were examined by reacting the enzyme (about 3 μ M) with varied concentrations of NADH (5–500 μ M). Absorbance changes associated with NADH binding to free enzyme were recorded at 355 nm [15]. Reactions performed in the presence of 130 μ M DACA (premixed with enzyme) were monitored at 464 nm [16]. The kinetics of NAD⁺ binding were similarly examined at 281 nm [15], using about 8 μ M enzyme and 50–200 μ M coenzyme.

The rate constant for coenzyme dissociation from the enzyme-NADH-DACA complex was estimated by displacement methods. An enzyme solution containing at least 93 % of the ternary complex (formed from 4 μ M enzyme, 10 μ M NADH, and 100 μ M DACA) was reacted with a large excess of NAD⁺ (3.2 mM). The displacement of NADH thus initiated was followed at the wavelength of maximum absorption of the enzyme-NADH-DACA complex (464 nm [16]). To avoid interferences from catalytic breakdown of this productive ternary complex, enzyme was preincubated with NADH in one syringe and mixed with DACA and NAD⁺ from the second syringe. Under such conditions (due to the high rate of association of DACA to the enzyme-NADH complex [16]), formation of the enzyme-NADH-DACA complex was found to be virtually completed within the dead-time of the stopped-flow apparatus, i.e. absorbance changes recorded can be assumed to reflect exclusively the subsequent slow displacement of NADH from the ternary complex.

The rate of dissociation of coenzyme from the binary enzyme-NADH complex was examined as detailed previously [17], except that measurements were made at 355 nm in the absence of pyrazole.

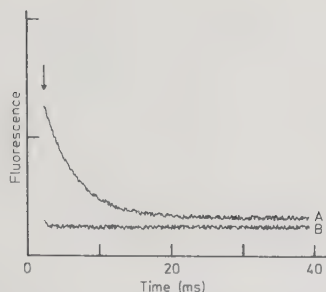


Fig. 1. Displacement of auramine O by DACA. Fluorescence changes recorded for the reaction of liver alcohol dehydrogenase (2 μM), pre-equilibrated with 20 μM auramine O, with 65 μM DACA in the absence (trace B) or presence (trace A) of 1 mM NADH (premixed with enzyme). Measurements performed at 25°C in 0.1 M ionic strength phosphate buffer, pH 9

RESULTS

DACA binding to the enzyme

The kinetics of complex formation between liver alcohol dehydrogenase and *trans*-*N,N*-dimethylaminocinnamaldehyde (DACA) were examined fluorimetrically by stopped-flow techniques, using auramine O as reporter ligand [14,18]. Measurements were performed at pH 9, where catalytic breakdown of the enzyme $\cdot$ NADH $\cdot$ DACA complex is negligibly slow [16] in comparison to rates of the binding processes examined. In a first series of experiments, the enzyme was preincubated with auramine O in the absence or presence of saturating concentrations of NADH. Auramine O was then displaced from the enzyme by the addition of varied concentrations of DACA. The typical results in Fig. 1 show that auramine O is displaced at a measurably low rate in the presence of NADH (trace A). The apparent first-order rate constant for the transient governing the displacement reaction was found to tend towards a limiting value of $250 (\pm 50) \text{ s}^{-1}$ with increasing concentrations of DACA. Since DACA is known to be rapidly and tightly bound to the enzyme $\cdot$ NADH complex [16], this value can be taken to provide a direct estimate of the rate constant for auramine O dissociation from the ternary enzyme $\cdot$ NADH $\cdot$ auramine O complex. The corresponding equilibrium dissociation constant was found to be $6 (\pm 1) \mu\text{M}$ under the experimental conditions used in the present work. This gives a value of about $40 \mu\text{M}^{-1} \text{ s}^{-1}$ for the rate constant for auramine O association to the enzyme $\cdot$ NADH complex, which agrees closely with the on-velocity constant estimates reported for DACA binding to the latter complex [16].

Fluorescence changes associated with the displacement of auramine O by DACA in the absence of NADH (trace B in Fig. 1) were at least 90% completed within the 2.3-ms dead-time of the stopped-flow apparatus, even at the lowest DACA concentration tested (30 μM). These reactions, therefore, cannot be rate-limited by any first-order transient slower than 1000 s^{-1} . Two inferences can be drawn from the latter observation, when interpreted in view of the transient-state kinetic theory for displacement reactions [17] and reported estimates of equilibrium dissociation constants for the binary enzymic complexes formed with auramine O (12 μM [19]) and DACA (35 μM [2]). Firstly, the rate constant for DACA

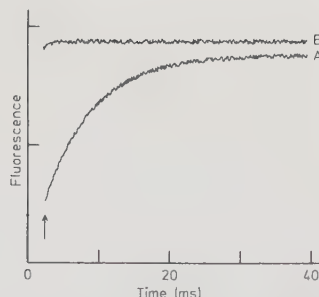


Fig. 2. Displacement of DACA by auramine O. Fluorescence changes recorded for the reaction of liver alcohol dehydrogenase (2 μM), pre-equilibrated with 20 μM DACA, with 400 μM auramine O in the absence (trace B) or presence (trace A) of 1 mM NADH (premixed with enzyme). Other conditions as in Fig. 1

association to free enzyme cannot be lower than $10 \mu\text{M}^{-1} \text{ s}^{-1}$. Secondly, the rate constant for auramine O dissociation must exceed 600 s^{-1} , which corresponds to an association rate constant exceeding $50 \mu\text{M}^{-1} \text{ s}^{-1}$.

The reverse displacement process was similarly examined in a second series of experiments. Enzyme solutions pre-equilibrated with DACA in the absence or presence of 1–5 mM NADH were reacted with sufficiently high (according to control experiments and results described above) concentrations of auramine O to ensure that the latter ligand equilibrates rapidly with both free enzyme and the enzyme $\cdot$ NADH complex. Fluorescence changes detectable by stopped-flow techniques in these experiments, therefore, can be assumed to reflect a measurably low rate of DACA dissociation from the enzyme.

Typical results, obtained with the highest auramine O concentration tested (400 μM), are given in Fig. 2. Trace A shows that DACA is displaced from the ternary enzyme $\cdot$ NADH $\cdot$ aldehyde complex in a measurably slow transient with a rate constant of $160 (\pm 20) \text{ s}^{-1}$. This value is in satisfactory agreement with the off-velocity constants of 220 – 350 s^{-1} determined photometrically for DACA binding to the enzyme $\cdot$ NADH complex [16]. The displacement of DACA from the binary enzyme $\cdot$ aldehyde complex (trace B) was found to be at least 96% completed within the dead-time of the instrument. Hence it follows that the rate constant for DACA dissociation from the latter complex (k_{-2} in Scheme 1) must exceed 1400 s^{-1} , which corresponds to a DACA association rate constant (k_2) exceeding $40 \mu\text{M}^{-1} \text{ s}^{-1}$.

These kinetic data establish that the stabilizing effect of NADH on DACA binding [2] derives mainly from a decreased rate of dissociation of the aldehyde at the ternary-complex level.

Effect of DACA on coenzyme association rates

The effect of DACA on the kinetics of NADH binding to liver alcohol dehydrogenase at pH 9 was examined by standard photometric stopped-flow measurements [17]. Fig. 3 shows the observed variation with coenzyme concentration of apparent first-order rate constants (r) for the transient governing NADH binding in the absence or presence of 130 μM DACA. In consistency with previous reports, r values deter-

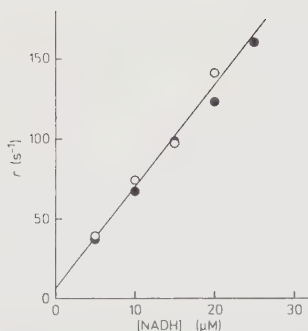


Fig. 3. Effect of DACA on the rate of NADH association to liver alcohol dehydrogenase. Apparent first-order rate constants (r) determined by stopped-flow techniques for the transient governing the reaction of varied concentrations of NADH with 2.9 μM enzyme in the absence (O) or presence (●) of 130 μM DACA (pre-equilibrated with enzyme). Other conditions as in Fig. 1

mined for NADH binding to free enzyme conform well to the linear relationship

$$r = k_{\text{on}}[\text{NADH}] + k_{\text{off}} \quad (1)$$

with $k_{\text{on}} = 7 \mu\text{M}^{-1} \text{s}^{-1}$ and $k_{\text{off}} = 6 \text{s}^{-1}$ [17]. The linear dependence of r on $[\text{NADH}]$ persists in the presence of DACA, and the k_{on} value for coenzyme binding is not detectably (within an experimental precision of about 10%) affected by the aldehyde. The effect of DACA on the k_{off} value cannot be reliably estimated from the results in Fig. 3. Control experiments performed with 130 μM DACA and 500 μM NADH showed that no first-order process slower than 1000s^{-1} limits the rate of formation of the enzyme·NADH·DACA complex.

Data reported in the preceding section establish that the equilibration between enzyme and 130 μM DACA may be considered as rapid in comparison to the observed rates of coenzyme binding. Under such conditions, the rate parameter r for the transient governing coenzyme binding according to Scheme 1 should be given by Eqn. (1) with

$$k_{\text{on}} = \frac{k_1}{1 + \frac{k_2[\text{DACA}]}{k_{-2}}} + \frac{k_4}{1 + \frac{k_{-2}[\text{DACA}]}{k_2}} \quad (2)$$

$$k_{\text{off}} = \frac{k_{-1}}{1 + \frac{k_3[\text{DACA}]}{k_{-3}}} + \frac{k_{-4}}{1 + \frac{k_{-3}[\text{DACA}]}{k_3}} \quad (3)$$

when catalytic breakdown of the productive ternary-complex is negligibly slow ($k \ll r$). Assuming that $k_{-2}/k_2 = 35 \mu\text{M}$ [2] and $k_{-3}/k_3 = 6 \mu\text{M}$ [16], Eqns. (2) and (3) become approximately

$$k_{\text{on}} = 0.21 k_1 + 0.79 k_4 \quad (4)$$

$$k_{\text{off}} = 0.04 k_{-1} + 0.96 k_{-4} \quad (5)$$

for 130 μM DACA, while they reduce to

$$k_{\text{on}} = k_1 \quad (6)$$

$$k_{\text{off}} = k_{-1} \quad (7)$$

in the absence of aldehyde. Interpreted in view of Scheme 1, therefore, results in Fig. 3 provide clear evidence that the magnitude of k_4 differs insignificantly (less than 15%) from

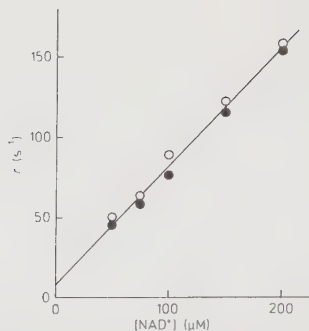


Fig. 4. Effect of DACA on the rate of NAD^+ association to liver alcohol dehydrogenase. Apparent first-order rate constants (r) determined for the transient governing the reaction of varied concentrations of NAD^+ with 8 μM enzyme in the absence (O) or presence (●) of 130 μM DACA. Other conditions as in Fig. 1

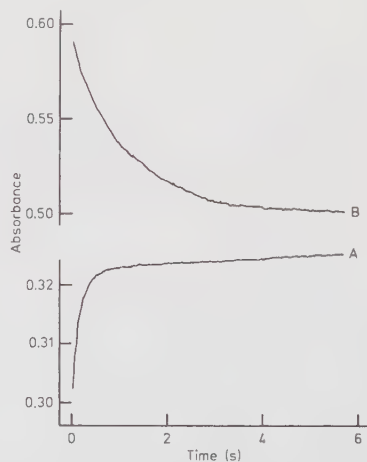


Fig. 5. Effect of DACA on the rate of NADH dissociation from liver alcohol dehydrogenase. Absorbance changes associated with the reaction of 4 μM enzyme, pre-equilibrated with 10 μM NADH, with 3.2 mM NAD^+ in the absence (trace A; recorded at 355 nm) or presence (trace B; recorded at 464 nm) of 100 μM DACA (premixed with NAD^+). Other conditions as in Fig. 1

that of k_1 , i.e. NADH association to the enzyme·DACA complex must occur at essentially the same rate as NADH association to free enzyme. This means (since $k_1 k_{-2} k_3 k_{-4} = k_{-1} k_2 k_{-3} k_4$) that the k_{off} value for coenzyme binding would be expected to decrease from 6s^{-1} in the absence of aldehyde [17] to about 1s^{-1} in the presence of 130 μM DACA. Data in Fig. 3 are consistent with that expectation.

The rate of NAD^+ association to liver alcohol dehydrogenase was similarly examined (Fig. 4) and found to remain essentially unaffected by complex formation between enzyme and DACA.

Effect of DACA on the NADH dissociation rate

Fig. 5 shows the absorbance changes observed when a large excess of NAD^+ was added to enzyme solutions con-

Table 1. Kinetic data for complex formation between liver alcohol dehydrogenase, NADH, and DACA by the mechanism in Scheme 1

Rate constant	Estimate	Reference
k_2	$> 40 \mu\text{M}^{-1} \text{s}^{-1}$	this work
k_3	$45 \mu\text{M}^{-1} \text{s}^{-1}$	[16]
k_4	$7 \mu\text{M}^{-1} \text{s}^{-1}$	this work
k_1	$7 \mu\text{M}^{-1} \text{s}^{-1}$	[17]
k_{-2}	$> 1400 \text{s}^{-1}$	this work
k_{-3}	270s^{-1}	[16]
k_{-4}	0.9s^{-1}	this work
k_{-1}	6s^{-1}	[17]

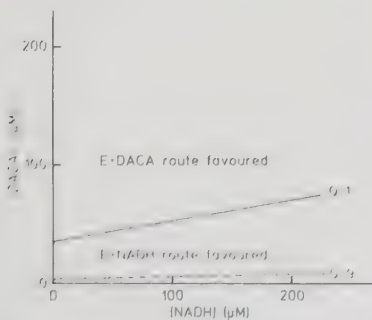


Fig. 6. Flow pattern for the enzymic reduction of DACA by NADH. Analysis based on Eqn. (8) and data in Table 1

taining at least 93 % of the enzyme · NADH complex (trace A; recorded at 355 nm) or the enzyme · NADH · DACA complex (trace B; recorded at 464 nm). The displacement of NADH occurs with an apparent first-order rate constant of $5.6 (\pm 0.7) \text{s}^{-1}$ in the absence of DACA, decreasing to $0.9 (\pm 0.1) \text{s}^{-1}$ in the presence of DACA. These values can be taken as estimates of the rate constant for NADH dissociation from the respective complexes (k_{-1} and k_{-4} in Scheme 1). The alternative possibility that weak or slow NAD^+ binding may contribute significantly to rate-limitation of the displacement reactions is ruled out by binding data reported previously [2, 17] and in Fig. 4.

Results in Fig. 5 provide direct evidence that the NADH dissociation rate decreases about sixfold on DACA binding to the enzyme. This confirms the conclusions drawn indirectly from data in Fig. 3.

Order of NADH and DACA binding

Utilization of the two alternative pathways for ternary-complex formation in Scheme 1 can be quantitatively characterized in terms of the quotient (Q) between reaction flow via the enzyme · NADH complex and reaction flow via the enzyme · aldehyde complex. This quotient is given [7] by

$$Q = \frac{k_1 k_3 (k_{-2} + k_4 [\text{NADH}])}{k_2 k_4 (k_{-1} + k_3 [\text{Ald}])} \quad (8)$$

under steady-state conditions, where Ald stands for aldehyde substrate. We will (arbitrarily) define the reaction as effectively ordered when one pathway accounts for more than 90 % of the total reaction flow ($Q > 9$ or $Q < 1/9$).

Results summarized in Table 1 provide the information required for analysis of the flow pattern governing formation of the catalytically productive enzyme · NADH · DACA complex. Fig. 6 illustrates the pattern obtained if magnitudes of k_2 and k_{-2} equal the minimum values now established. The full line in Fig. 6 represents the combinations of NADH and DACA concentrations for which the two pathways in Scheme 1 are equally utilized ($Q = 1$). The region above (below) this line corresponds to coenzyme and aldehyde concentrations for which $Q < 1$ ($Q > 1$), i.e. for which ternary-complex formation proceeds predominantly via the enzyme · DACA (enzyme · NADH) complex. The region below the dashed line in Fig. 6 indicates NADH/DACA concentrations giving $Q > 9$, such that the reaction may be considered as effectively ordered with coenzyme binding preceding DACA binding. If k_2 (and hence also k_{-2}) is larger by a certain factor than the minimum value $40 \mu\text{M}^{-1} \text{s}^{-1}$ now obtained, slopes of lines drawn in Fig. 6 will decrease by the same factor while intercepts remain unaffected.

Since DACA can be used at concentrations up to $300 \mu\text{M}$ substrate and coenzyme binding during the enzymic reduction of this aldehyde by NADH cannot be generally characterized as compulsory, effectively, or even preferentially ordered. The flow pattern in Fig. 6 demonstrates that both pathways for ternary-complex formation are of catalytic significance. The actual combination of substrate and coenzyme concentrations used will determine whether the catalytic reaction proceeds predominantly via one pathway or the other. This characterizes the order of substrate and coenzyme binding as entirely random.

Three of the binding steps in Scheme 1 (NADH binding to free enzyme [1, 17], aldehyde binding to free enzyme [2], and aldehyde binding to the enzyme · NADH complex [16, 20]) can be considered as essentially pH-independent between pH 6 and 9. This means that the fourth binding step must be so too. Although referring to measurements made at pH 9, therefore, results in Table 1 and Fig. 6 provide a satisfactory (at least for the purpose of the present investigation) description of the binding properties of the enzyme over the pH range 6–9.

DISCUSSION

Synergism between NADH and DACA binding

The interaction of liver alcohol dehydrogenase with the chromophoric substrate *trans-N,N*-dimethylaminocinnamaldehyde (DACA) has been exceptionally well characterized [2, 16, 21–23]. In particular, rate constant estimates are available from previous reports [16, 17] for all reaction steps leading to formation (or dissociation) of the enzyme · NADH · DACA complex by the enzyme · NADH pathway in Scheme 1. The present results provide the corresponding information about the enzyme · aldehyde pathway for formation of the enzyme · NADH · DACA complex (Table 1). The kinetic properties of the two pathways can now be compared, which makes it possible to consider the mechanistic problems mentioned in the introduction.

We then wish to discuss firstly the synergism between NADH and DACA binding to the enzyme. Recent equilibrium measurements have shown that coenzyme and DACA are about six times more tightly bound in the ternary enzyme · NADH · DACA complex than in the respective binary complexes [2]. This heterotropic cooperativity effect, the presence and magnitude of which is confirmed by the kinetic

data in Fig. 3 and 5, is associated with a mechanistically important change in the binding interactions between enzyme and DACA. The catalytically crucial inner-sphere coordination of DACA to the active-site zinc ion [16,22,23] is not detectably at hand in the binary enzyme · DACA complex, but requires the presence of bound coenzyme. We have attributed observed effects of NADH on aldehyde binding and bonding to hydrophobic substrate-coenzyme-site interactions resulting in dehydration of the active-site region [2]. According to this idea, synergistic stabilization of the enzyme · NADH · aldehyde complex should be achieved mainly through decreased rates of dissociation of the two ligands. Results summarized in Table 1 provide clear evidence that the positive cooperativity in the binding of NADH and DACA has such a kinetic origin. There is no detectable effect of DACA binding on the coenzyme association rate ($k_1 \approx k_4$), and the rate of DACA association is not significantly enhanced on NADH binding ($k_3 \leq k_4$).

These observations establish that the enzyme · aldehyde pathway for ternary-complex formation is fully kinetically competent in the sense that it is not impaired or blocked through low rates of substrate and/or coenzyme association. When the reaction proceeds by this pathway, the coenzyme association step must include concomitant ligand exchange at the catalytic zinc ion (such that DACA is transferred from the outer to the inner coordination sphere of the metal with displacement of zinc-bound water [16,22]). Results in Fig. 3 demonstrate that such ligand exchange is rapid enough not to affect the kinetics of coenzyme association over the concentration ranges now examined.

DACA binding to the enzyme · NADH complex can be analogously envisaged to proceed minimally by a two-step mechanism, formation of an outer-sphere enzyme · NADH · DACA complex preceding ligand exchange at the catalytic zinc ion resulting in inner-sphere metal coordination of the aldehyde. The present results, and kinetic data reported by Dunn et al. [16], establish that formation of the enzyme · NADH · DACA complex (irrespective of what pathway is utilized) cannot be rate limited by any first-order process slower than 1000 s^{-1} . Conformational changes induced by NADH binding or otherwise associated with ternary-complex formation [22,23], as well as ligand exchange at the catalytic zinc ion, must show rate constants exceeding this value. This confirms our previous conclusion [24] that the observed limitation at about 250 s^{-1} of the rate constant for complex formation between enzyme and zinc-chelating reagents cannot be attributed to a slow step of water dissociation from the catalytic zinc ion [25,26]. Ligand exchange at the zinc ion is probably rate-limited by dissociation of zinc-bound water, but such dissociation would be expected to show a rate constant several orders of magnitude higher than 1000 s^{-1} [21].

It follows from these considerations that DACA binding to the enzyme · NADH complex can be anticipated to proceed by a slow step of (outer-sphere) aldehyde association followed by a rapidly equilibrating step of ligand exchange at the catalytic zinc ion. This means that the step of ligand exchange will contribute only to the off-velocity constant (k_{-3} in Scheme 1) for DACA binding, while the on-velocity constant (k_3) can be assumed to reflect exclusively the primary association process leading to formation of the outer-sphere enzyme · NADH · DACA complex. This explains the present observation that rate constants for the association of auramine O (which does not combine to catalytic zinc on complex formation with the enzyme [19]) and DACA to the enzyme · NADH complex are

of closely agreeing magnitude. As pointed out by Dunn et al. [16], the latter association reaction is rapid enough to be rate-limited mainly by diffusional factors, and the same obviously applies for DACA binding to free enzyme (Table 1). Complex formation with NADH leads to significant conformational changes of the enzyme, but these changes do not appreciably affect the geometry or accessibility of the substrate-binding site [1,23]. It seems reasonable to assume, therefore, that aldehyde association proceeds at essentially the same diffusion-limited rate whether or not the coenzyme-binding site is occupied ($k_2 \approx k_3$).

General characteristics of NADH and aldehyde binding

According to the above discussion, results in Table 1 can be taken to indicate that effects of NADH on the kinetics of DACA binding are analogous to the observed effects of DACA on the kinetics of NADH binding. Complex formation with one ligand appears to have no pronounced effect on the association rate of the other. This kinetic pattern is probably characteristic for the formation of enzyme · NADH · aldehyde complexes in general. DACA may be an atypical substrate inasmuch as it is exceptionally tightly bound and forms a productive ternary complex with exceptionally low catalytic reactivity [16]. The low rate of catalytic hydride transfer from NADH to DACA can be related to electronic properties of the substrate which are of little significance for its binding, however, and does not reflect substrate binding in an atypical (low-productive) mode. On the contrary, it has been well established that DACA is bound at the same site and through similar bonding interactions as aldehydes in general [2,22]. If the coenzyme association process is assumed to be generally affected by aldehyde binding at this site, then such effects should be particularly pronounced with DACA which interacts exceptionally strongly with the site. The observation (Fig. 3) that DACA has no detectable effect on the NADH, therefore, renders the possibility extremely unlikely that other aldehydes will have such an effect.

On similar grounds, the observation that bound NADH does not prevent DACA from combining to the enzyme at what appears to be a diffusion-limited rate can be taken to indicate that aldehyde association rate constants are typically of the same order of magnitude as those determined for DACA association and are probably not much affected by NADH binding ($k_2 \approx k_3 > k_1$). Hence it follows also that aldehydes can generally be assumed to dissociate much more rapidly than coenzyme from the enzyme · NADH · aldehyde complex ($k_{-3} \gg k_{-4}$). The quotient k_{-3}/k_{-4} equals 300 with DACA as the substrate (Table 1) and should be considerably larger with other aldehydes since DACA is exceptionally tightly bound.

Results now obtained with DACA, therefore, lead to the inference that the formation of enzyme · NADH · aldehyde complexes can be assumed to proceed typically by Scheme 1 with $k_1 \approx k_4$, $k_2 \approx k_3$, and $k_{-3} \gg k_{-4}$. The possibility that aldehydes may associate more rapidly to free enzyme than to the enzyme · NADH complex ($k_2 > k_3$) cannot be definitely excluded, but the assumption that $k_2 \approx k_3$ seems more reasonable and is conservative with respect to all main conclusions drawn below.

Catalytic significance of enzyme · aldehyde complexes

Heterotropic cooperativity in ligand binding to enzymes has usually been found to reflect effects on ligand-dissociation

rather than ligand-association rates. The present conclusion that such a kinetic pattern applies for NADH and aldehyde binding to liver alcohol dehydrogenase may seem trivial, therefore, but is of interest as it implies that the rate of substrate and coenzyme association to the enzyme is independent of the binding order. This renders questionable the widely held view that enzymic aldehyde reduction proceeds by an effectively ordered mechanism with preferential utilization of the enzyme · NADH pathway for ternary-complex formation. Assuming that $k_1 \approx k_4$ and $k_2 \approx k_3$ (see preceding section), Eqn. (8) reduces approximately to

$$Q = \frac{k_{-2} + k_1 [\text{NADH}]}{k_{-1} + k_2 [\text{Ald}]} < \frac{k_{-2}}{k_2 [\text{Ald}]} + \frac{k_1 [\text{NADH}]}{k_2 [\text{Ald}]} \quad (9)$$

showing that reaction flow via the enzyme · aldehyde pathway may actually predominate ($Q < 1$) if aldehyde concentrations exceed those required for half-saturation of free enzyme. Recently reported equilibrium binding data establish that such concentrations are experimentally accessible [2] and indicate that several kinetic studies of liver alcohol dehydrogenase have been performed over concentration ranges where NADH/aldehyde binding cannot possibly be characterized as effectively or even preferentially ordered.

For example, Kvassman and Pettersson examined the enzymic reduction of 0.02–20 mM benzaldehyde by 60 μM NADH at pH 8.75 [27]. Benzaldehyde is less tightly bound to free enzyme ($k_{-2}/k_2 = 6 \text{ mM}$ [2]) than is DACA, but would not be expected to associate more slowly than DACA to the enzyme. Assuming that $k_2 > 40 \mu\text{M}^{-1} \text{ s}^{-1}$ for benzaldehyde binding (it actually suffices to assume that $k_2 > 1 \mu\text{M}^{-1} \text{ s}^{-1}$), it follows from Eqn. (9) that utilization of the enzyme · aldehyde pathway in the experiments of Kvassman and Pettersson varied from less than 0.4% to more than 75% of the total reaction flow. In the reduction of 18 mM benzaldehyde ($3 \times k_{-2}/k_2$), about 1 M NADH ($2000000 \times k_{-1}/k_1$) would probably be required to render utilization of the enzyme · aldehyde pathway catalytically insignificant ($Q > 9$). It would seem extremely inappropriate to consider such reactions as ordered with coenzyme binding preceding substrate binding. Binary enzyme · aldehyde complexes are undoubtedly of catalytic significance.

The present results, therefore, lead to the conclusion that the enzymic reaction mechanism must be characterized as random with respect to the order of NADH and aldehyde binding during catalysis. The flow pattern governing the enzymic reduction of DACA by NADH (Fig. 6) appears to be qualitatively representative for aldehyde reduction in general.

Kinetic significance of enzyme · aldehyde complexes

Our conclusion that NADH and aldehydes are bound in random order is in obvious conflict with standard interpretations of the observed rate behaviour of liver alcohol dehydrogenase [1, 3–6]. This calls for an analysis of the implications of the present results with respect to the steady-state rate (v) of enzymic aldehyde reduction by the simplified random-order mechanism in Scheme 1. Extrapolation of the analysis to more elaborate reactions schemes, or to the transient reaction state [28], is straightforward and leads to no major modification of conclusions now drawn.

Assuming that $k_1 \approx k_4$, $k_2 \approx k_3$, and $k_{-3} \gg k_{-4}$ (see preceding discussion), the complex full rate equation for Scheme 1 will reduce effectively to the Dalziel [3] relationship

$$\frac{1}{v} = \phi_0 + \frac{\phi_1}{[\text{NADH}]} + \frac{\phi_2}{[\text{Ald}]} + \frac{\phi_{12}}{[\text{NADH}][\text{Ald}]} \quad (10)$$

as soon as substrate dissociation from the enzyme · aldehyde complex is rapid in comparison to the rate-limiting step(s) of the reaction ($k_{-2} \gg 1/\phi_0$) [7]. Such conditions undoubtedly obtain with all aldehyde substrates hitherto examined, which explains why aldehyde reduction has never been found to exhibit deviations from Michaelis-Menten kinetics typical of substrate inhibition or substrate activation. Generalized relationships for ϕ_1 and ϕ_2 [other coefficients in Eqn. (10) are of no diagnostic interest in the present context] are given [8] by

$$\phi_1 = \frac{k_{-4}}{k} \cdot \left[\frac{1}{k} + \frac{1}{k_{-3} + k_{-4}} \right] + \frac{1}{k_1} \cdot \left[\frac{k_{-3}}{k_{-3} + k_{-4}} \right]^2 \quad (11)$$

$$\phi_2 = \frac{k_{-3}}{k_3} \cdot \left[\frac{1}{k} + \frac{1}{k_{-3} + k_{-4}} \right] + \frac{1}{k_2} \cdot \left[\frac{k_{-4}}{k_{-3} + k_{-4}} \right]^2 \quad (12)$$

With the above assumptions they reduce effectively to

$$\phi_1 = \frac{k_{-4}}{k_4 k} + \frac{1}{k_1} \quad (13)$$

$$\phi_2 = \frac{k_{-3} + k}{k_3 k} \quad (14)$$

showing that two main kinetic cases may obtain depending on the relative rates of catalytic decomposition of (k) and coenzyme dissociation from (k_{-4}) the productive ternary complex. When $k_{-4} \gg k$ it follows that $k_{-3} \gg k$ and Eqns. (13) and (14) reduce to

$$\phi_1 = \frac{k_{-4}}{k_4 k} \quad (15)$$

$$\phi_2 = \frac{k_{-3}}{k_3 k} \quad (16)$$

The rate behaviour of the reaction system will then agree with that predicted for a rapid-equilibrium random-order mechanism [29]. When $k_{-4} \ll k$, however, it follows from the assumption $k_1 \approx k_4$ that Eqn. (13) becomes

$$\phi_1 = \frac{1}{k_1} \quad (17)$$

and aldehyde reduction by Scheme 1 will conform to a rate equation which is experimentally indistinguishable from the one [29] derived for a compulsory-order mechanism with NADH binding preceding binding of the aldehyde.

Rapid-equilibrium conditions ($k_{-4} \gg k$) might obtain for the enzymic reduction of DACA at high pH, where the catalytic activity of the enzyme · NADH · DACA complex is extremely low [16]. Catalytic hydride transfer in productive ternary complexes formed with more typical aldehyde substrates invariably has been found to occur at rates ($k > 100 \text{ s}^{-1}$ [30, 31]) which greatly exceed the expected rate ($k_{-4} < k_{-1} = 6 \text{ s}^{-1}$ [2, 17]) of coenzyme dissociation from the complex. The condition $k_{-4} \ll k$, therefore, can be anticipated to be fulfilled for aldehyde reduction in general. This confirms and explains the observations made in previous kinetic studies of liver alcohol dehydrogenase. The enzyme behaves kinetically as if aldehyde reduction took place exclusively via the

enzyme · NADH pathway in Scheme 1. Furthermore, and this is the most interesting implication of the above analysis from a theoretical point of view, the enzyme will show such a rate behaviour over any range of substrate and coenzyme concentrations, including those where the reaction actually proceeds predominantly via the enzyme · aldehyde pathway. The binding order indicated by the rate equation (according to standard interpretations) is not necessarily the one which is actually preferred.

General conclusions

The present investigation provides an unexpected answer to the much-discussed question of why liver alcohol dehydrogenase reactions are effectively ordered for substrate and coenzyme concentrations within the Michaelis-Menten range: there is no reason to believe that they are, at least not with respect to the enzymic reduction of aldehyde substrates. The latter reaction is governed by a rate equation indistinguishable from the one expected for a compulsory-order mechanism with NADH binding preceding aldehyde binding, but the above kinetic analysis demonstrates that this type of rate equation may obtain also for a random-order mechanism and over concentration ranges where the converse binding order predominates. Observations that reactions proceeding by a ternary-complex mechanism conform to a compulsory-order type of rate equation cannot be taken as evidence that the actual reaction is correspondingly ordered (not even preferentially).

Information about the actual partitioning of reaction flow between the two pathways for ternary-complex formation in Scheme 1 can be obtained only by examination of the flow quotient Q defined by Eqn. (8). Kinetic results and flow analyses now reported for liver alcohol dehydrogenase establish that the enzymic reduction of aldehydes must be assumed to involve random (i.e. substrate and coenzyme concentration-dependent) utilization of both of the alternative pathways. This conclusion is fully consistent with (in fact supported by) the isotope exchange data presented by Silverstein et al. [32] and Ainslie et al. [33]. Reaction flow via the enzyme · NADH pathway may certainly have predominated with the substrates (e.g. acetaldehyde which is exceptionally weakly bound to free enzyme [2]) and over the concentration ranges examined in many kinetic studies of the enzyme, but it seems obvious that enzymic aldehyde reduction in several instances has been studied under such conditions that the enzyme · aldehyde pathway has been the preferred one. Binary enzyme · aldehyde complexes do not contribute detectably to the rate behaviour of the enzyme, but may nevertheless provide most significant contributions to the catalytic reaction flow.

These results draw attention to the inadequacy of conventional steady-state kinetic studies for the purpose of establishing the order of substrate (or substrate and coenzyme) binding in enzyme reactions proceeding by a ternary-complex mechanism. Partitioning of reaction flow between the alternative pathways for ternary-complex formation in such systems is not dependent on the rate of catalytic decomposition of the ternary complex, but the type of rate equation which obtains is very much so. This means that there is no obvious correlation between the flow quotient and the rate behaviour of the system [7,8]. Reactions may well exhibit deviations from Michaelis-Menten kinetics (e.g. substrate activation or inhibition), or conform to a rate equation of the rapid-equilibrium random-order type, over concentration ranges where reaction flow is restricted effectively to one of the alternative pathways.

Conversely, as the present kinetic analysis demonstrates, reactions may be governed by a rate equation of the compulsory-order type corresponding to exclusive utilization of one of the pathways irrespective of which pathway is actually preferred. This lack of correlation between rate behaviour and substrate binding order in ternary-complex systems has not been generally recognized, but should obviously always be considered in the mechanistic interpretation of kinetic data obtained with such systems. Pathways which do not contribute to the rate equation may carry a major part of the catalytic reaction flow and intermediates of such pathways may accumulate in most significant (e.g. spectroscopically) amounts.

These implications of the present and previous kinetic analyses lead to the conclusion that it is of no mechanistic interest *per se* to establish the so-called 'kinetic mechanism' for enzymic ternary-complex reactions, i.e. to decide whether the reactions are random or in any way ordered as judged from the type of rate equation which obtains. Such characterization of the reaction kinetics is of interest only when it leads to unambiguous interpretation of coefficients in the rate equation [cf. Eqns. (11–17)] and thus provides information about rate constants for reaction steps which do contribute to the rate behaviour of the system. In the latter respect, there is no doubt that the rate equation for liver alcohol dehydrogenase catalysis of aldehyde reduction by NADH has been correctly characterized as being of the type corresponding to compulsory binding of coenzyme prior to substrate.

The present results, therefore, do not call for any reinterpretation of reported steady-state or transient-state kinetic parameter values for the enzymic reduction of aldehyde substrates, at least not of values determined in the absence of reactants showing a high affinity for the enzyme · aldehyde complexes. It could be of importance to recognize in other practical or theoretical contexts, however, that reaction flow via the enzyme · aldehyde pathway may be most significant and even predominant under certain conditions.

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Synergism between coenzyme and alcohol binding to liver alcohol dehydrogenase

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1. Heterotropic cooperativity effects in the binding of alcohols and NAD^+ or NADH to liver alcohol dehydrogenase have been examined by equilibrium measurements and stopped-flow kinetic studies. Equilibrium data are reported for benzyl alcohol, 2-chloroethanol, 2,2-dichloroethanol, and trifluoroethanol binding to free enzyme over the pH range 6–10.

2. Binary-complex formation between enzyme and alcohols leads to inner-sphere coordination of the alcohol to catalytic zinc and shows a pH dependence reflecting the ionization states of zinc-bound water and the zinc-bound alcohol. The affinity of the binding protonation state of the enzyme for unionized alcohols increases approximately by a factor of 10 on complex formation between enzyme and NAD^+ or NADH .

3. The rate and kinetic cooperativity with coenzyme binding of the alcohol association step indicates that enzyme-bound alcohols participate in hydrogen bonding interactions which affect the rates of alcohol and coenzyme equilibration with the enzyme without providing any pronounced contribution to the net energetics of alcohol binding.

4. The pK_a values determined for alcohol deprotonation at the binary-complex level are linearly dependent on those of the free alcohols, and can be readily reconciled with the pK_a values attributed to ionization of zinc-bound water. Alcohol coordination to catalytic zinc provides a major contribution to the pK_a shift which ensures that the substrate is bound predominantly as an alcoholate ion in the catalytically productive ternary complex at physiological pH. The additional pK_a shift contributed by NAD^+ binding is less pronounced, but may be of particular mechanistic interest since it increases the acidity of zinc-bound alcohols relatively to that of zinc-bound water.

Liver alcohol dehydrogenase [1] catalyzes alcohol/aldehyde interconversion by a ternary-complex mechanism with NAD^+/NADH as coenzymes. It appears well established that alcohol and aldehyde substrates are directly coordinated to the catalytic zinc ion of the enzyme subunit in the productive ternary complexes formed during catalysis [1, 2], but there is no detectable inner-sphere metal coordination of aldehydes at the binary-complex level [3]. The mechanistically crucial ligation of aldehydes to catalytic zinc, therefore, must be induced by cooperative substrate-coenzyme interactions. The strength, kinetic effects, and mechanistic implications of these cooperative interactions have been characterized in recent studies of the synergism between coenzyme and aldehyde binding to the enzyme [3, 4].

The present investigation was undertaken to characterize similarly the synergism between coenzyme and alcohol binding to liver alcohol dehydrogenase, information which is required for detailed evaluation of the role played by NAD^+ in the chemical activation of alcohol substrates. The kinetics of coenzyme binding to the enzyme have been previously examined in sufficient detail [1, 5], as has the interaction of the enzyme- NAD^+ complex with benzyl alcohol [6], trifluoroethanol [7], 2-chloroethanol, and 2,2-dichloroethanol [8]. We have now carried out kinetic and equilibrium studies of the binding of these alcohols to free enzyme, as well as of coenzyme binding to binary enzyme-alcohol complexes. The results provide mechanistically interesting information in several

respects, but are discussed primarily with regard to the mechanism and energetics of the catalytic step of substrate deprotonation in the enzymic oxidation of alcohols by NAD^+ .

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [9], except that 50 mM phosphate buffer was used for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [10] and are reported throughout as active-site concentrations. The coenzymes NAD^+ and NADH (Sigma Chemical Corp., grade III quality) were purified as described by Gurr et al. [11]. Other reagents were of highest available quality and used without further purification.

All experiments reported in the present paper were carried out at 25 °C in 0.1 M ionic strength phosphate (pH 6–8), pyrophosphate (pH 8.8), or carbonate (pH 9–10) buffer, prepared using twice-distilled water.

Methods

Equilibrium constants for alcohol binding to liver alcohol dehydrogenase were determined fluorimetrically as described by Sigman and Glazer [12] and further detailed by Andersson et al. [13]. Fluorescence changes associated

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

with binding of the reporter ligand auramine O to the enzyme were monitored at 530 nm (excitation at 430 nm) using a Perkin-Elmer MPF-2A spectrofluorimeter. Solutions containing about 0.3 μM auramine O and varied concentrations of alcohol were titrated with enzyme in the absence or presence of ligands which combine to the coenzyme-binding site of the enzyme subunit. Concentrations of the latter ligands were high enough [0.2 mM NADH, 2 mM NAD^+ , 2 mM ADP-ribose, 5 mM AMP, 6 mM $\text{Pt}(\text{CN})_2^{2-}$] to be more than 97% saturating according to previously reported binding data [5, 14]. Dissociation constant estimates reported in Fig. 1 and 2 and Table 2 represent the mean of at least duplicate independent determinations, standard deviations of the estimates being typically about 15% of the values given.

Effects of benzyl alcohol and trifluoroethanol on complex formation between enzyme and 2,2'-bipyridine were examined with a Shimadzu UV-240 spectrophotometer, alcohol dissociation equilibrium constants being calculated as described by Sigman [15]. Effects of the two alcohols on the kinetics of coenzyme binding to the enzyme were studied by stopped-flow techniques, using the rapid-reaction equipment and methods for data evaluation detailed previously [4]. Absorbance changes associated with the binding of NADH and NAD^+ were monitored at 355 nm and 281 nm, respectively, and the alcohols were preincubated with enzyme (5–10 μM) prior to mixing in the stopped-flow apparatus.

Rate constants for alcohol dissociation from the binary enzyme · trifluoroethanol complex were determined by transient-state kinetic displacement methods [5] with auramine O as the displacing reagent and reporter ligand [4]. Fluorescence changes associated with auramine O binding were recorded by stopped-flow techniques. The excitation wavelength was 430 nm, and a Corning CS-3-69 filter was used to avoid registration of light emitted at wavelengths below 470 nm. Reactions were initiated by mixing a solution containing 6 μM enzyme and 10 mM trifluoroethanol with a solution containing varied concentrations of auramine O (100–500 μM). Under such conditions, auramine O binding to free enzyme is virtually completed within the 2.3 ms dead-time of the stopped-flow instrument [4], and the fluorescence changes recorded can be attributed to auramine O binding to the enzyme · trifluoroethanol complex, i.e. to displacement of the alcohol from the complex. Estimates of the trifluoroethanol dissociation rate constant were obtained by extrapolation to infinite auramine O concentration of the apparent first-order rate constants calculated for the transient governing the displacement reaction [5].

The transient-state kinetics of enzymic benzyl alcohol oxidation by NAD^+ were examined by stopped-flow techniques as described by Kvassman and Pettersson [16].

RESULTS

Alcohol binding to free enzyme

The interaction of liver alcohol dehydrogenase with alcohols was studied by standard fluorimetric equilibrium methods, using auramine O as a reporter ligand [12, 13]. All alcohols examined were found to be bound competitively with the reporter ligand, and equilibrium constants ($K_{\text{E,Alc}}$) for dissociation of the binary enzyme · alcohol complexes formed with benzyl alcohol, 2-chloroethanol, 2,2-dichloroethanol, and trifluoroethanol were determined at different pH between 6 and 10. The results in Fig. 1 and 2 show that there is no

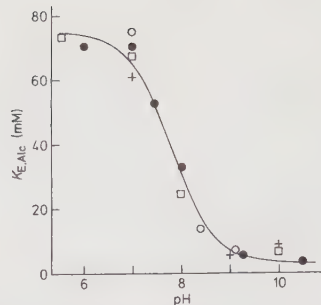


Fig. 1. Effect of pH on trifluoroethanol binding to liver alcohol dehydrogenase. Equilibrium constants ($K_{\text{E,Alc}}$) for dissociation of the binary enzyme · alcohol complex determined at 25 °C in 0.1 M ionic strength buffer solutions using auramine O (●), bipyridine (○), NAD^+ (□), or NADH (+) as reporter ligand. The curve drawn represents a least-squares fit of Eqn (1) to data obtained by the auramine O method, calculated with the assumption that $\text{p}K_9 = 9.2$.

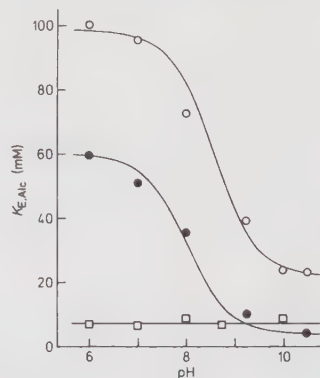
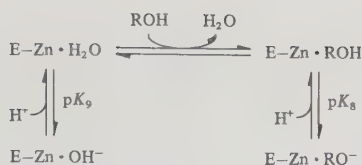


Fig. 2. Effect of pH on alcohol binding to liver alcohol dehydrogenase. Equilibrium constants ($K_{\text{E,Alc}}$) for dissociation of the binary enzyme · alcohol complexes formed with 2-chloroethanol (○), 2,2-dichloroethanol (●), and benzyl alcohol (□), determined at 25 °C in 0.1 M ionic strength buffer solutions using auramine O as reporter ligand

significant effect of pH on benzyl alcohol binding, whereas complex formation with the halogenated ethanol derivatives exhibits a pH dependence analogous to that observed for alcohol binding to the enzyme · NAD^+ complex [6–8].

Results in Fig. 1 and 2, therefore, were interpreted in view of Scheme 1, which is analogous to the reaction scheme proposed by Kvassman and Pettersson for alcohol binding in the presence of NAD^+ [6]. According to Scheme 1, alcohols combine to the catalytic zinc ion of the enzyme subunit with concomitant displacement of zinc-bound water. Ligand exchange at the metal ion is assumed to be prevented by ionization of the zinc-bound water or alcohol molecule with acid dissociation constants K_9 and K_8 , respectively. Equilibrium analysis of Scheme 1 then gives

$$K_{\text{E,Alc}} = K_{\text{E,Alc}}^* \cdot \frac{1 + \frac{K_9}{[\text{H}^+]}}{1 + \frac{K_8}{[\text{H}^+]}} \quad (1)$$



Scheme 1. Mechanism accounting for the observed pH dependence of alcohol (ROH) binding to liver alcohol dehydrogenase

where $K_{\text{E,Alc}}^*$ denotes the pH independent equilibrium constant for the actual alcohol dissociation step. Eqn (1) prescribes that $K_{\text{E,Alc}}$ may vary more or less strongly with pH depending on the relative magnitudes of K_8 and K_9 , and can thus be used also for description of the pH independence of benzyl alcohol binding ($K_8 = K_9$).

Regression analysis of data in Fig. 1 and 2 with Eqn (1) as regression function gave estimates of $\text{p}K_9$ ranging from 9.0 to 9.3 for the three halogenated alcohols tested. These values differ insignificantly from each other and from the $\text{p}K_a$ value of 9.2 previously ascribed to ionization of zinc-bound water in free enzyme [5, 17, 18], consistent with the predictions of Scheme 1. Regression curves drawn in Fig. 1 and 2, therefore, were calculated with the assumption that $\text{p}K_9 = 9.2$ for all alcohols, and the corresponding best-fit estimates of $K_{\text{E,Alc}}^*$ and $\text{p}K_8$ are given in Table 1 and Fig. 3, respectively. The Brönsted plot in Fig. 3 shows that the experimentally determined $\text{p}K_8$ values are linearly dependent on the $\text{p}K_a$ value for ionization of the respective free alcohol with a Brönsted α value of 0.50.

Binary-complex formation between enzyme and the above alcohols was found to be competitive also with 2,2'-bipyridine binding to the active-site zinc ion. Estimates of $K_{\text{E,Alc}}$ determined from the observed effects of the alcohols on bipyridine binding agreed within experimental precision with those obtained using auramine O as reporter ligand (Fig. 1).

Effect of coenzymes on alcohol binding

Fig. 4 shows the effect of NAD^+ on the affinity of liver alcohol dehydrogenase for alcohols now considered, as calculated from results in the preceding section and previously reported estimates of the equilibrium constant ($K_{\text{EO,Alc}}$) for alcohol dissociation from the ternary enzyme $\cdot \text{NAD}^+ \cdot$ alcohol complexes [6–8]. Data in Fig. 4 have been extrapolated to the pH range 3–11 in order to illustrate the interplay of the ionization steps which affect the magnitude of $K_{\text{E,Alc}}$ and $K_{\text{EO,Alc}}$. Over the pH range 6–10 where data are firmly based on experimental observations, the cooperativity factor ($K_{\text{E,Alc}}/K_{\text{EO,Alc}}$) for synergistic stabilization of the ternary complex varies from 10 to 6000 depending on pH and alcohol structure. At neutral pH, NAD^+ binding can be anticipated to lead to an at least fortyfold increase in affinity of the enzyme for alcohols in general (Fig. 4), an effect which may be of obvious physiological importance.

The $\text{p}K_a$ of zinc-bound water has been reported to increase from 9.2 to 11.2 on NADH binding [19, 20], and the $\text{p}K_a$ of zinc-bound alcohols should be similarly affected. NADH binding would thus be expected to shift the main pH dependence of alcohol binding to pH values above 9, and attempts were made to detect such a pH dependence. Equilibrium constants ($K_{\text{ER,Alc}}$) for alcohol dissociation from the ternary enzyme $\cdot \text{NADH} \cdot$ alcohol complexes, determined over the pH range 8–10 by the auramine O method, showed

Table 1. Alcohol binding to liver alcohol dehydrogenase

Equilibrium constants determined for the dissociation of unionized alcohols from complexes formed with the binding protonation state of the enzyme in the absence ($K_{\text{E,Alc}}^*$) or presence of saturating concentrations of NADH ($K_{\text{ER,Alc}}$) or NAD^+ ($K_{\text{EO,Alc}}^*$ [6–8]). Standard deviations of the estimates are 10–20% of the values given

Alcohol	$K_{\text{E,Alc}}^*$ mM	$K_{\text{ER,Alc}}$	$K_{\text{EO,Alc}}^*$
Trifluoroethanol	75	6	8
2,2-Dichloroethanol	60	8	6
2-Chloroethanol	100	25	21
Benzyl alcohol	7	0.8	0.7

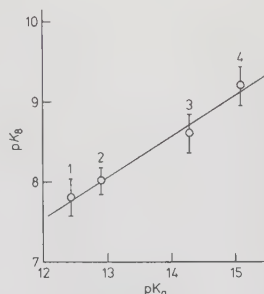


Fig. 3. Dependence of $\text{p}K_8$ for ionization of the binary complexes formed between liver alcohol dehydrogenase and alcohol ligands on the $\text{p}K_a$ of the free alcohol. Brönsted plot of $\text{p}K_8$ values determined from data in Fig. 1 and 2 vs the $\text{p}K_a$ value for ionization of trifluoroethanol (1), 2,2-dichloroethanol (2), 2-chloroethanol (3), and benzyl alcohol (4). The slope of the regression line drawn corresponds to a Brönsted α value of 0.50

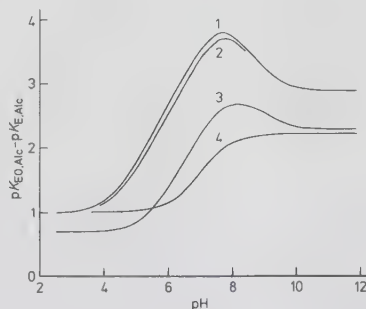


Fig. 4. Effect of pH on the cooperativity factor for NAD^+ and alcohol binding to liver alcohol dehydrogenase. Logarithmic plot of the cooperativity factor $K_{\text{E,Alc}}/K_{\text{EO,Alc}}$ vs pH, calculated from data in Fig. 1 and 2 and previously reported data for the interaction of the enzyme $\cdot \text{NAD}^+ \cdot$ complex [6–8] with trifluoroethanol (1), 2,2-dichloroethanol (2), 2-chloroethanol (3), and benzyl alcohol (4)

no significant variation with pH, however. The pH-independent $K_{\text{ER,Alc}}$ estimates obtained are given in Table 1. They lead to the conclusion that (over the examined pH range) NADH binding increases the affinity of the enzyme for benzyl alcohol by a factor of 9 independently of pH, whereas

Table 2. Effect of complex formation at the coenzyme-binding site on alcohol binding to liver alcohol dehydrogenase

Equilibrium constants ($K_{\text{EL,Aic}}$) for alcohol dissociation from different enzyme · ligand · benzyl alcohol complexes determined by the auramine O method in 0.1 M ionic strength pyrophosphate buffer, pH 8.8. The estimate obtained kinetically with NAD^+ as the ligand [6] is included for comparison and refers to the interaction of unionized benzyl alcohol with the binding protonation state of the enzyme

Ligand (L)	$K_{\text{EL,Aic}}$ mM	$K_{\text{EL,Aic}}/K_{\text{E,Aic}}$
Absent	7	1.0
$\text{Pt}(\text{CN})_4^{2-}$	6	1.2
AMP	4.2	1.7
ADP-ribose	3.7	1.9
NADH	0.8	9
NAD^+	0.7	10

Table 3. Effect of pH on the rate of alcohol dissociation from liver alcohol dehydrogenase

Rate constants (k_{off}) determined by displacement methods in 0.1 M ionic strength carbonate buffer for trifluoroethanol dissociation from the binary enzyme · alcohol complex

pH	k_{off} s^{-1}
9.0	> 1400
9.5	1050 (± 200)
10.0	330 (± 50)
10.5	120 (± 15)

cooperativity factors for the binding of NADH and other alcohols examined may vary approximately from 10 to 0.5 depending on pH.

Table 2 shows that complex formation between enzyme and coenzyme-competitive ligands such as ADP-ribose, AMP, and $\text{Pt}(\text{CN})_4^{2-}$ results only in weak (less than twofold) tightening of the binding of benzyl alcohol at pH 8.8. This indicates that the nicotinamide portion of the coenzyme provides a major contribution to the more pronounced synergistic effects observed with NADH and NAD^+ .

Trifluoroethanol dissociation rate

The binary enzymic complexes formed with alcohols in Table 1 were found to dissociate rapidly, and stopped-flow kinetic estimates of alcohol dissociation rate constants (k_{off}) could be obtained only with trifluoroethanol at high pH. The k_{off} values thus determined (Table 3) show the approximate proportionality to hydrogen concentration prescribed by Scheme 1 and by the estimated pK_a value of 7.8 for ionization enzyme-bound trifluoroethanol. Data in Table 3 correspond to an off-velocity (on-velocity) constant of about 50000 s^{-1} ($6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) for binding of trifluoroethanol in its unionized state, which may be compared to the values of 500 s^{-1} ($6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) reported for trifluoroethanol binding to the enzyme · NAD^+ complex [7]. It may be concluded from these data that NAD^+ binding to the enzyme has a pronounced effect on the association as well as the dissociation rate of the unionized alcohol ligand, and that the alcohol association rate is much too low to be diffusion controlled in the absence as well as the presence of enzyme-bound coenzyme.

Effect of alcohols on coenzyme binding

The effect of trifluoroethanol on the kinetics of complex formation between enzyme and NAD^+ or NADH was examined by stopped-flow techniques at different pH between 6 and 10. The typical results in Fig. 5 show that the apparent on-velocity constant for NAD^+ binding at pH 8 decreases (from $9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ in the absence of alcohol [5]) to $7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of 300 mM trifluoroethanol. Assuming that $K_{\text{E,Aic}} = 30 \text{ mM}$ at pH 8 (Fig. 1), this NAD^+ association rate decrease agrees within experimental precision with that expected for a competitive effect of the alcohol, i.e. NAD^+ association to the enzyme · alcohol complex does not contribute detectably to the observed rate of coenzyme binding and must hence be at least one order of magnitude slower than NAD^+ association to free enzyme. Similar results were obtained over the entire pH range examined. $K_{\text{E,Aic}}$ values calculated from such data with the assumption that the trifluoroethanol effect is practically competitive are included in Fig. 1 and agree with those determined using auramine O or bipyridine as reporter ligands.

The rate of NADH association to the enzyme between pH 6 and 10 was analogously found to be competitively affected by trifluoroethanol, as well as by benzyl alcohol (Fig. 6), effects of the two alcohols being consistent with the respective binding data reported in Fig. 1 and 2.

Substrate inhibition of the transient rate of enzymic benzyl alcohol oxidation

According to the transient-state rate equations derived for a compulsory-order mechanism by Pettersson [21], the apparent first-order rate constant (r'_1) for the transient governing NADH production during liver alcohol dehydrogenase catalysis of alcohol oxidation by NAD^+ [16] should conform to the substrate-independent relationship

$$\frac{1}{r'_1} = \frac{1}{r'_{1,\infty}} + \frac{\phi'_1}{[\text{NAD}^+]} \quad (2)$$

at saturating substrate concentrations; $r'_{1,\infty}$ represents the limiting value of r'_1 at saturating substrate and coenzyme concentrations, and ϕ'_1 denotes the Dalziel coefficient describing the NAD^+ concentration dependence of the steady-state reaction velocity. Eqn (2) should apply whether or not the steady-state reaction exhibits substrate inhibition due to the formation of abortive enzyme · NADH · alcohol complexes [22, 23], but if the substrate forms a dead-end complex with free enzyme the approximate relationship

$$\frac{1}{r'_1} = \frac{1}{r'_{1,\infty}} + \frac{\phi'_1}{[\text{NAD}^+]} \cdot \left(1 + \frac{[\text{Aic}]}{K_{\text{E,Aic}}}\right) \quad (3)$$

can be shown to obtain at high alcohol concentrations.

Fig. 7 shows the effect of high concentrations of substrate on the transient-state kinetics of enzymic benzyl alcohol oxidation by NAD^+ at pH 7. The dashed line in Fig. 7 corresponds to Eqn (2) and represents the limiting relationship towards which $1/r'_1$ tends with increasing non-inhibitory concentrations (less than 1 mM [23]) of benzyl alcohol [16]. Experimental data in Fig. 7 demonstrate the presence of an inhibitory effect of substrate which can be well described in terms of Eqn (3), and which corresponds to a $K_{\text{E,Aic}}$ value (9 mM) agreeing satisfactorily with that reported for benzyl alcohol in Table 1. Besides confirming the mechanistic significance of the enzyme-alcohol interactions examined with auramine O and bipyridine as reporter ligands, results in Fig. 7

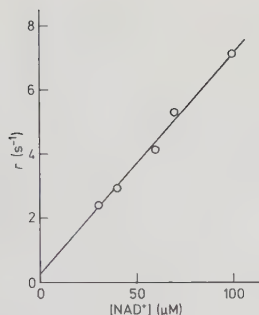


Fig. 5. Effect of trifluoroethanol on the kinetics of NAD^+ binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) for the transient governing NAD^+ binding to the enzyme determined in 0.1 M ionic strength phosphate buffer, pH 8, containing 300 mM trifluoroethanol

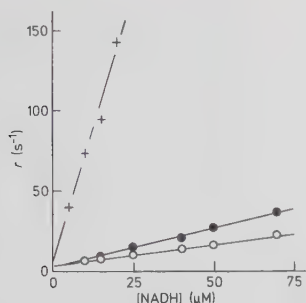


Fig. 6. Effect of alcohols on the kinetics of NADH binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) for the transient governing NADH binding to the enzyme determined in 0.1 M ionic strength carbonate buffer, pH 9.0, in the absence of alcohols (+) and in the presence of 100 mM benzyl alcohol (●) or 200 mM trifluoroethanol (○)

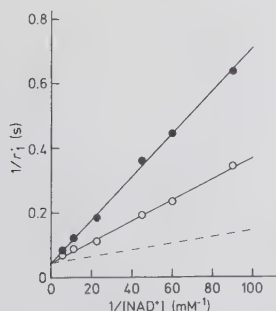


Fig. 7. Inhibitory effect of high substrate concentrations on the transient rate of enzymic benzyl alcohol oxidation by NAD^+ . Apparent first-order rate constants (r_1) determined at pH 7 for the transient governing the conversion of NAD^+ into NADH during liver alcohol dehydrogenase catalysis of the oxidation of 50 mM (●) or 20 mM (○) benzyl alcohol

provide clear evidence that the binary enzyme · benzyl alcohol complex does not react at a detectable rate with NAD^+ . This corroborates the observations made with the inhibitor trifluoroethanol (Fig. 5) and establishes that the corresponding negative effects on NAD^+ binding are at hand also with the substrate benzyl alcohol.

It is of methodological interest to note that the substrate inhibition effect revealed by transient-state kinetic data in Fig. 7 cannot be readily detected by steady-state kinetic measurements [22, 23]. Due to the synergism between alcohol and NADH binding (Table 1), substrate inhibition reflecting binary-complex formation will become significant only over concentration ranges where the steady-state reaction is already strongly inhibited by formation of the abortive enzyme · NADH · benzyl alcohol complex.

DISCUSSION

Binding site for alcohols

The catalytic zinc ion of the liver alcohol dehydrogenase subunit is bound at the bottom and junction of two deep pockets which constitute, respectively, the substrate-binding site and the nicotinamide-binding part of the coenzyme-binding site [1]. Auramine O interacts specifically with the substrate-binding site at some distance from catalytic zinc [1, 13, 15], while bipyridine forms a chelate with the active-site metal ion [1]. The present results establish that alcohols combine competitively with auramine O to the substrate-binding site in their interaction with free enzyme, as has been previously reported for aldehyde substrates [3]. In contrast to aldehyde binding [3], however, binary-complex formation with alcohols now examined is competitively affected also by bipyridine and shows a pH dependence reflecting the ionization state of zinc-bound water and the ionization properties of the enzyme-bound ligand (Fig. 1–3). These observations provide clear evidence that alcohol binding to free enzyme, analogously with alcohol binding to the enzyme · NAD^+ complex [6–8], results in direct ligation of the alcohol to catalytic zinc. The difference between aldehydes (which do not combine to catalytic zinc [3]) and alcohols in their bonding interactions at the binary-complex level can be well understood in view of the different intrinsic affinities of these ligands for metal ions.

The observation that there is no detectable effect of pH on $K_{\text{ER, Alc}}$ over the pH range 8–10 is consistent with reported effects of NADH on the acidity of zinc-bound water [19, 20] and can be taken to indicate that alcohols ionize with $\text{p}K_a$ values above 10 in the enzyme · NADH · alcohol complexes. The possibility that alcohols do not combine detectable to catalytic zinc in such complexes is rendered extremely unlikely by our previous finding that NADH binding drastically promotes inner-sphere zinc coordination of aldehyde substrates [3].

Heterotropic cooperativity in the equilibrium binding of coenzymes and alcohols

Equilibrium data reported in the present paper establish that the synergism between coenzyme and alcohol binding to liver alcohol dehydrogenase shows a comparatively complex dependence on pH, alcohol structure, and coenzyme oxidation state (cf. Fig. 4). The synergistic pattern can be rationalized in simple terms, however, when related to the three mechanistic events (two ionization steps in addition to the actual step of

alcohol binding) which control the affinity of the enzyme for alcohols according to Scheme 1. The effect of coenzymes on the acidity of zinc-bound water has been previously discussed in detail [17–20], and the effect of coenzymes on the ionization properties of zinc-bound alcohols will be considered in the final section of this paper. The effect of NADH and NAD^+ on the actual step of alcohol binding is illustrated by the equilibrium data in Table 1, which refer to the interaction of unionized alcohols with enzyme species containing zinc-bound water in the neutral state. Binding of the unionized alcohols becomes approximately one order of magnitude tighter in the presence of oxidized as well as reduced coenzyme. The nicotinamide portion of the coenzyme appears to be of major importance for induction of this synergistic effect (Table 2).

These results are closely similar to those obtained with aldehyde ligands and indicate that complex formation between enzyme and NAD^+ or NADH stabilizes the binding of unionized alcohols through hydrophobic coenzyme-substrate-site interactions analogous to those proposed to account for the observed synergism between coenzyme and aldehyde binding [3]. This explains why the synergistic effects are comparatively weak, strongly dependent on binding of the nicotinamide portion of the coenzyme, but otherwise more closely related to site occupation than to the structure and oxidation state of the ligands; it may be noted that NADH stabilizes the binding of fatty acid amides [24], aldehydes [3], and unionized alcohols (Table 1) to approximately the same extent. Attribution of the coenzyme-induced stabilization of the actual step of alcohol binding to hydrophobic interactions is consistent also with crystallographic evidence showing that the formation of enzyme $\cdot \text{NAD}^+$ $\cdot$ alcohol complexes leads to an extensive dehydration of the active-site region [2, 25].

Data in Table 1 establish that the enzyme $\cdot \text{NADH}$ complex shows essentially the same affinity for unionized benzyl alcohol as for benzaldehyde [3] and that such is the case also for free enzyme despite the difference in metal coordination state of alcohol and aldehyde substrates at the binary-complex level. This indicates that inner-sphere coordination to catalytic zinc does not contribute appreciably to the net energetics of substrate binding or to the synergistic stabilization of enzyme $\cdot$ coenzyme $\cdot$ aldehyde complexes. More convincing evidence in that direction is provided by the recent observations that the stability of the enzyme $\cdot \text{NAD}^+$ $\cdot$ dimethylaminocinnamaldehyde complex remains unaffected by pH [3] over a pH range (6–10) where the chromophoric properties of the complex exhibit drastic changes with pH reflecting the extent to which the bound aldehyde may compete successfully with water (hydroxide ion) for the inner coordination sphere of the active-site zinc ion [26].

Heterotropic cooperativity in the kinetics of coenzyme and alcohol binding

The present results point to some fundamental differences in the kinetics of interaction of alcohols and aldehydes with liver alcohol dehydrogenase. The synergistic stabilization of enzyme $\cdot$ coenzyme $\cdot$ aldehyde complexes appears to originate mainly from effects of one ligand on the dissociation rate of the other [4], but the binding of coenzymes and alcohols shows a cooperativity reflecting effects on ligand association as well as dissociation rates. Furthermore, aldehyde association to free enzyme [4] and to catalytic zinc in the enzyme $\cdot \text{NADH}$ complex [27] occurs at a rate which is high enough to be considered as diffusion-controlled. Alcohol association in the

presence [6–8] or absence (Table 3) of bound coenzyme, however, is much too slow to be rate-limited by diffusional factors, even at pH values where the enzyme is predominantly in its binding protonation state. These observations provide clear evidence that alcohols participate in a rate-limiting protein-ligand interaction which is not at hand with aldehyde ligands and which, therefore, must involve the alcohol hydroxyl group without being primarily related to the coordinative interaction of this group with catalytic zinc. The only reasonable explanation for such a difference in the binding of the ligands is that the alcohol hydroxyl group, but not the aldehyde carbonyl group, participates in hydrogen bonding interactions with the enzyme.

Crystallographic evidence has been presented which indicates that alcohols [25], but not aldehydes [28], are hydrogen-bonded to the hydroxyl group of Ser-48 in catalytically component enzyme $\cdot$ coenzyme $\cdot$ substrate complexes. It was further concluded from such evidence [25] that coenzyme binding installs a proton relay system linking the hydroxyl group of zinc-bound alcohols to solvent through a series of hydrogen bonds involving the side chain of Ser-48, a nicotinamide ribose hydroxyl group of the coenzyme, and the imidazole group of His-51 (cf. Scheme 2). Results now obtained in solution are consistent with the crystallographic data and indicate that the structural rearrangements associated with formation of the alcohol/Ser-48 hydrogen bond limit the alcohol association rate and hence must account also for the observed effects of NAD^+ on alcohol association rates (Tables 3). The converse effects of alcohols on coenzyme association (Fig. 5–7) can be similarly explained.

Eklund et al. [25] suggested that insertion of the coenzyme's hydroxyl group into the proton relay system of hydrogen bonds may promote the binding of alcohols and thus explain why the enzyme has a kinetically ordered mechanism. Data in Fig. 4 confirm that NAD^+ binding increases the affinity of the enzyme for alcohols more or less strongly, but this synergistic equilibrium effect must be considered as neutral with regard to the kinetic order of ligand association since alcohol binding increases the affinity of the enzyme for NAD^+ to exactly the corresponding extent. Furthermore, the absence of a pronounced difference in the affinity of free enzyme and of the enzyme $\cdot \text{NADH}$ complex for unionized benzyl alcohol (Table 1) and benzaldehyde [3] indicates that hydrogen bonding to Ser-48 provides no major contribution to the net energetics of alcohol binding; the system of hydrogen bonds appears to affect mainly the rate at which the enzyme equilibrates with coenzyme and substrate. In the latter respect, data Table 3 provide clear evidence for a negative effect of NAD^+ on the trifluoroethanol association rate, attributable partly to the unfavourable perturbation of the pK_a of zinc-bound water [20] and partly to an approximately tenfold decrease in magnitude of the on-velocity constant for the actual step of alcohol association. Since the latter effect must be related to the coenzyme's interaction with Ser-48, and since there seem to be no positive effects of coenzymes on aldehyde association rates [4], NAD^+ binding is extremely unlikely to promote the association of any alcohol to the enzyme and to render the catalytic reaction ordered through such a mechanism.

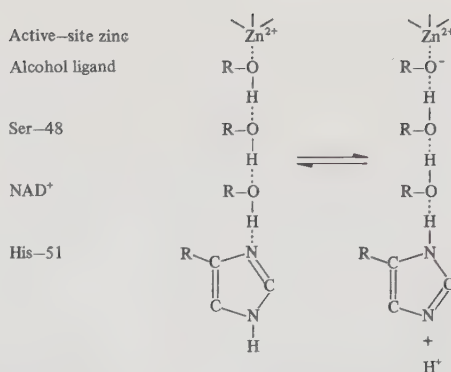
Attention rather should be drawn to the observation that complex formation between enzyme and tightly bound alcohols such as trifluoroethanol and benzyl alcohol leads to a most pronounced decrease in the coenzyme association rates (Fig. 5–7). That effect can be shown by flow analysis [4] to have a direct impact on the kinetic order of ligand binding

and to strongly disfavour utilization of the enzyme·alcohol pathway for ternary-complex formation. It should be noted, however, that NAD^+ and NADH association rates recently were found to remain essentially unaffected by complex formation between enzyme and ethanol [29]. This means that the kinetic alcohol-coenzyme cooperativity pattern may differ considerably for different alcohols, such that the enzyme·alcohol pathway may be fully catalytically competent with certain substrates and more or less kinetically impaired with others. Dalziel et al. [30] early concluded from steady-state kinetic measurements that the enzymic oxidation of alcohols by NAD^+ must be assumed to proceed by a random-order mechanism. The present results do not call for any major modification of that conclusion.

Ionization properties of zinc-bound alcohols

Studies of the pH dependence of liver alcohol dehydrogenase catalysis have established that the productive enzyme· NAD^+ ·alcohol complex participates in a mechanistically crucial ionization step which affects the rate of hydride ion transfer from substrate to NAD^+ [8, 16] as well as the rates of ligand dissociation from the ternary complex [6–8]. Kvassman and Pettersson [16] proposed that these effects of pH reflect an obligatory step of alcoholate ion formation at the ternary-complex level, attributable to a kinetically coupled acid-base system involving minimally the zinc-bound alcohol and the side chains of Ser-48 and His-51. According to the crystallographic results of Eklund et al. [25], this ionization step can be formally represented as in Scheme 2 at physiological pH where His-51 is likely to be mainly unprotonated.

Although it now appears well established that the zinc-bound alcohol forms part of the ionizing system which accounts for the observed effects of pH on alcohol binding and oxidation [8], several authors have preferred to relate these pH effects primarily to deprotonation of His-51 [25, 31–33]. This may be justified in the sense that His-51 (being the only component of the ionizing system in Scheme 2 that is in direct contact with solution [25]) is likely to be transiently protonated/deprotonated during the step of alcohol ionization. It would not seem reasonable, however, to attribute the kinetically determined pK_a values to deprotonation of His-51 [31, 32], because such deprotonation *per se* cannot account for the drastic kinetic effects from which the pK_a values have been determined. Ionization of the pK_a -4.3 group in the enzyme· NAD^+ ·trifluoroethanol complex [7], for example, decreases the rate of alcohol dissociation from the complex by a factor exceeding 50 000 (corresponding to an energetic transition state difference exceeding 28 kJ/mol). This effect is undoubtedly much too strong to derive from inductive changes of the alcohol/Ser-48 hydrogen bonding interaction, and is even less likely to reflect changes in the hydrophobic interactions of the alcohol with the enzyme. The effect can only be reasonably explained in terms of changes in the alcohol-zinc interaction, and must hence be attributed to ionization of the alcohol ligand itself. Reported pH dependence data for the reactivity of various enzyme· NAD^+ ·alcohol complexes [6–8, 16] must then be taken to provide evidence for the absence of a detectable thermodynamic coupling of the ionizations of zinc-bound alcohols and His-51, consistent with the conclusion in the preceding section that the alcohol/Ser-48 hydrogen bond does not contribute significantly to the net energetics of alcohol binding. This means that zinc-bound alcohols and His-51 may be regarded as essentially independent ionizing groups, the latter probably showing a pK_a within



Scheme 2. Alcohol ionization step accounting for the kinetically observed effects of pH on the reactivity of enzyme· NAD^+ ·alcohol complexes. The system of hydrogen bonds shown is likely to mediate proton transfer between the zinc-bound alcohol and solution at physiological pH

Table 4. Effect of liver alcohol dehydrogenase on the ionization properties of alcohols

pK_a values determined for alcohol (Alc) ionization in solution [34, 35] and in binary (Fig. 3) or ternary [6–8] enzyme·alcohol complexes

Alcohol	Estimated pK_a value for			pK_a shift induced by	
	Alc	E·Alc	E· NAD^+ ·Alc	enzyme	NAD^+
Trifluoroethanol	12.4	7.8	4.3	4.6	3.5
2,2-Dichloroethanol	12.9	8.0	4.5	4.9	3.5
2-Chloroethanol	14.3	8.6	5.4	5.7	3.2
Benzyl alcohol	15.1	9.2	6.4	5.9	2.8
Water	15.6	9.2	7.6	6.4	1.6

the range 6–7 and the former showing the pK_a values determined from the kinetic reactivity of different enzyme· NAD^+ ·alcohol complexes.

Similar considerations obviously apply for the interpretation of pH dependence data now reported. The analogy between the observed effects of pH on alcohol binding to free enzyme and to the enzyme· NAD^+ complex strongly indicates that pK_a values estimated from data in Fig. 1 and 2 are attributable to ionization of the enzyme-bound alcohols. The free energy correlation demonstrated by the Brønsted plot in Fig. 4 would seem definitely to establish the validity of that interpretation of the results, and Table 4 summarizes the information which is now available on the ionization properties of alcohols (free and enzyme-bound) considered in the present investigation.

Examination of Table 4 shows that the pK_a values for alcohol ionization decrease by 4.6–5.9 on formation of the binary enzyme·alcohol complexes. These pK_a shifts can be assumed to derive predominantly from the electrostatic field effect of the active-site zinc ion on the acidity of the metal-bound alcohols [36], and the magnitudes of the pK_a shifts are in excellent agreement with the pK_a value of 9.2 assigned to zinc-bound water in free enzyme [5, 17, 18]. Bertini et al. [37] recently suggested that the pK_a -9.2 dependence of the binding properties of liver alcohol dehydrogenase may reflect de-

protonation of the δ -NH group of the zinc-ligand His-67 rather than of zinc-bound water, but that possibility is rendered unlikely by quantum mechanistic data showing that zinc-bound water is intrinsically more acid than a zinc-bound imidazole molecule [38]. Furthermore, there is crystallographic evidence indicating that the His-67 δ -NH group is hydrogen bonded to the carboxyl group of Asp-49 [1, 39, 40]. The latter bonding interaction can be anticipated effectively to prevent ionization of His-67, suggesting that the enzyme is structurally designed to avoid the negative effects of such ionization on the Lewis acid strength of the active-site zinc ion and hence on the catalytic reactivity of the enzyme. The assignment of a pK_a of 9.2 to zinc-bound water in free enzyme, on the other hand, is fully consistent with the structural properties of the catalytic zinc site [19], based on exceptionally strong experimental evidence [1, 17–20], and corroborated by the present demonstration that zinc-bound alcohols exhibit analogously shifted pK_a values at the binary-complex level. NAD^+ binding to the enzyme causes an additional decrease in pK_a of the zinc-bound alcohols by 2.8–3.5 (Table 4), as might be expected from the well-documented stabilizing effect of NAD^+ on anion (including hydroxide ion) binding to catalytic zinc [1, 20]. The coenzyme-induced pK_a perturbation shows a correlation with the electron-withdrawing effect of the alcohol substituents, consistent with the suggestion [6] that inductive charge delocalization may render the stabilizing effect of NAD^+ stronger for zinc-bound alcoholate ions than for a zinc-bound hydroxide ion.

The present results, therefore, lend strong support to the mechanistic proposal of Kvassman and Pettersson [6], according to which the main effects of pH on liver alcohol dehydrogenase catalysis derive from interactions which serve the catalytic purpose of activating the alcohol substrate for hydride ion transfer by converting it into an alcoholate ion. The active-site zinc ion provides a major contribution to the pK_a perturbation which facilitates such activation of the substrate in the catalytically productive enzyme $\cdot NAD^+$ alcohol complex, but the less pronounced electrostatic field effect of NAD^+ may be of particular mechanistic interest since it affects alcohol and water ionization differently (Table 4). The combined effects of catalytic zinc and NAD^+ on the acidity of the zinc-bound ligands appear to be optimized to ensure that (at physiological pH) the substrate is bound predominantly as alcoholate ion in the productive ternary complex at the same time as zinc-bound water remains predominantly unionized in the substrate-binding enzyme species. The catalytic advantages of such a mechanistic arrangement have been discussed elsewhere [6, 8].

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